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ADHERENCE OF CHICKEN LYMPHOCYTES TO SURFACES

by



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## Abstract

A specific adherence technique has been developed and used to detect alloantigens and receptors of phytomitogens or plant lectins on the cell membrane of chicken lymphocytes. The term "allofixation" has been introduced to designate the specific adherence of chicken lymphocytes induced by alloantibodies. In contrast to specific adherence induced by either alloantibodies or plant lectins, cells adhere aspecifically in serum-free media. Allofixation requires fresh plasma whereas aspecific adherence is inhibited by the presence of serum or plasma. In the presence of serum or plasma, some plant lectins induce cell adherence. Phytohemagglutinin (PHA) induces cell adherence and agglutination, Concanavalin A (Con A) induces only cell adherence and Pokeweed mitogen (PWM) induces neither. The presence of agglutinins in PHA and Con A is probably responsible for this adherence. The cell adherence induced by plant lectins is inhibited by those glycosides which inhibit agglutination by these lectins.

Allofixation is capable of detecting antigens present on erythrocytes, and lymphocytes derived from peripheral blood, thymus or the bursa of Fabricius. The reaction is genetically specific as determined by blood typing (hemagglutination). Suitable conditions for the reaction are a pH of 8.5 and a period of 2 hours at room temperature. Divalent cations are required; the chelating agent, EDTA, completely inhibits the reaction. B alloantigens have been detected on the peripheral lymphocytes of chicken and on half of the bursa cells, but not on thymus lymphocytes. Some thymus specific antigens are detected on the peripheral lymphocytes as well as thymus cells, but not



on the bursa cells. The A and B specific reactions of peripheral lymphocytes differ. Gene dosage effects have been detected for  $\underline{B}^2$ ,  $\underline{A}^2$  and  $\underline{A}^6$  alleles, but not for  $\underline{B}^1$ ,  $\underline{B}^{13}$ ,  $\underline{B}^{14}$  or  $\underline{B}^{15}$ . Twice as many homozygous  $B_2$ ,  $A_2$  and  $A_6$  cells react by comparison with the heterozygotes. It was not possible to demonstrate a role of carbohydrate in B reactions, or a requirement for normal metabolism during these reactions.

The adherence of chicken lymphocytes can be induced by mammalian anti-chicken-sera plus fresh chicken plasma. Chicken anti-mouse-serum plus fresh chicken plasma induces the fixation of mouse lymph node cells. In these instances adherence is associated with lysis at high concentrations of antibody, or cytotoxicity at intermediate concentrations of antibody. IgG appears to be the principal source of allofixing antibody, but allofixation by primary antisera is sensitive to mercaptoethanol, indicating that some allofixing antibodies may be IgM.





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## Introduction

"Lymphocyte" is merely a term applied to a cell which is small and has a characteristic pachychromatic nucleus and a thin rim of cytoplasm lacking distinct granules and staining a pale blue in a blood smear (Astaldi and Lisiewicz, 1971; Elves, 1972; Metcalf and Moore, 1971; Nossal and Ada, 1971; Yoffey, 1967). Cells of such morphology are located in the peripheral blood, thymus, bursa of Fabricius of chicken, spleen, bone marrow and lymph nodes of mammals, and in other areas of the so-called peripheral lymphoid organs or tissues. This way of categorizing a cell simply by its morphology provides little or no information about its potential for differentiation or function. Recently, through the use of selective staining, isotope, chromosome and antigen markers, isolation, irradiation, and single-cell immuno-assays it has become clear that this morphological group of cells is functionally heterogenous. They can be separated into immunologically competent cells and those without such function, into long-lived and short-lived cells, into rapidly dividing cells and those dividing slowly, into cells which are multipotent and those which are unipotent, into cells with theta antigen on their surface and cells without that antigen, into cells capable of binding complement-antigen-antibody complex to their surfaces and cells unable to do so and into cells with one or another type of immunoglobulin surface receptor.

The objective of this study is twofold. Firstly, it is aimed at improving the present method of typing lymphocytes for their surface antigens. Secondly, it attempts to separate lymphocytes





according to the antigen specificities of their cell surfaces by immunoadherence to inert surfaces in the presence of antibodies. Immobilization of cells at inert surfaces would fulfill both of these objectives provided the immobilized cells are still active and functionally intact after interaction with the specific agents that immobilized them. These objectives have been achieved. The technique has been termed "allofixation." In addition to developing and applying "allofixation" I have compared it with lymphocyte immobilization in the absence of serum proteins, and in the presence of plant lectins.



## Chapter 1

### Lymphocyte Adherence Test

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## Introduction

This chapter deals with the methodology of immobilization of lymphocytes at surfaces by alloantibodies and with the aspecific adherence of cells in serum-free media. The conditions of the test and the application of the test in detecting alloantigens on lymphoid cells of different genetic lines and lymphoid organs of chicken are examined. The alloantigens detected by this method are compared with those detected by lymphoagglutination and immunofluorescence. Alloantigens present on the lymphocytes of chicken have been detected by these two methods. Using lymphoagglutination, Schierman and Nordskog (1962) were able to detect B and C blood group antigens on chicken lymphocytes, and David et al. (1966) and Davidenas (1970) detected B antigens by immunofluorescence. The A, D, and L blood group antigens could not be detected by agglutination (Schierman and Nordskog, 1962) and A antigens could not be detected by immunofluorescence (Davidenas, 1970). In addition to these tests for blood group antigens there is evidence that chicken lymphocytes bear alloantigens which may not be present on erythrocytes. McDermid (1968) and Thein and Schmid (1968) have reported lymphoagglutination with antisera which did not agglutinate erythrocytes. While following the early reaction of lymphocytes to alloantisera specific for B antigens we detected a loss of lymphocytes which was not due to lysis. The lost lymphocytes were recovered from the walls of the container, apparently undamaged. We present evidence that the rate and extent of adherence of small lymphocytes to glass and plastic can be used to detect A and B and antigens not detected on



erythrocytes. Therefore, we have developed a new technique based on the immobilization of lymphocytes at surfaces by their specific allo-antibodies. This method appears to be more sensitive than the methods previously reported for the detection of alloantigens on chicken lymphocytes.







## Materials and Methods

### I. Experimental Animals

Six lines of White Leghorn chickens were used in this study. Lines are designated according to their B genotypes, i.e.,  $\underline{B}^1/\underline{B}^1$ ,  $\underline{B}^2/\underline{B}^2$ ,  $\underline{B}^{13}/\underline{B}^{13}$ ,  $\underline{B}^{14}/\underline{B}^{14}$ ,  $\underline{B}^{15}/\underline{B}^{15}$ , and  $\underline{B}^{21}/\underline{B}^{21}$ . In addition, the first four B lines are also controlled at the A locus, e.g.,  $\underline{B}^2/\underline{B}^2$ ,  $\underline{A}^2/\underline{A}^2$ , and  $\underline{B}^2/\underline{B}^2$ ,  $\underline{A}^6/\underline{A}^6$  (sublines) and so on. The other blood groups are not controlled in our population of chickens. These B alleles were originally derived from chickens provided by Hy-Line Poultry Farms, Johnston, Iowa.

### II. Preparation of Lymphocytes

The problems of isolating pure lymphocytes have hampered the attempts to study them. Populations of lymphocytes often contain other cell types that may account for certain functions or transformations attributed to lymphocytes. Nonetheless, there are a number of methods that yield pure or almost pure concentrations of lymphocytes. Thoracic duct cannulation provides lymphocyte-rich cell suspensions. Teased lymphoid organs such as thymus or bursa pressed through a stainless steel mesh give similar preparations. The lymphocyte concentration in such suspensions may be enriched by removing other cells. Erythrocytes may be eliminated by hypotonic shock. Alternatively, erythrocytes may be preferentially agglutinated and removed by differential centrifugation. Such suspensions may then be passed through columns containing glass wool, or allowed to settle on the floor of a tissue culture dish. Lymphocytes tend not to adhere to surfaces and pass through glass wool and remain free floating after being placed in a tissue culture dish.



Macrophages, polymorphonuclear leukocytes (PMN), and platelets or thrombocytes, on the other hand, being sticky, adhere to the fibers of glass wool or the floor of the dish and are thereby removed from suspension.

(a) Isolation of Lymphocytes from Peripheral Blood

The isolation of lymphocytes from blood of chickens was originally reported by Terasaki (1959). The method of isolation was modified as described below. Blood was taken from a wing vein with a 2.5 to 3.0 ml. syringe (with 21 gauge needle) containing 0.2 ml. of heparin solution (100 U.S.P. units/ml. in Hanks' Balanced Salt Solution, HBSS) and transferred to a 12 x 75 mm plastic tube. This was centrifuged at 300 rpm (14 g) for 5 minutes and the buffy coat was transferred to a second tube. Only the upper lymphocyte-rich layer of the buffy coat was transferred since the lower layers of the plasma contained more granulocytes, monocytes, thrombocytes and erythrocytes than lymphocytes. Heparin was used as anticoagulant because blood collected with sodium citrate gave a high percentage of granulocytes and thrombocytes and there was a tendency for gel formation in the test system when citrated plasma was used. Disodium ethylenediaminetetraacetate (EDTA) was not used because the chelating agent inhibited the performance of the test. The lymphocyte-rich plasma was centrifuged at 500 rpm (39 g) and essentially cell-free plasma was removed and combined with the cell-free plasma from the first tube for later use. The centrifuged lymphocytes remaining in the second tube were suspended in 4 ml. of HBSS without washing since washing away of the trace amounts of serum or plasma will





produce unnecessary adhesion of cells to the wall of the container. The cell suspension was then incubated at 37°C in a 60 x 15 mm petri dish. Under these conditions the lymphocytes did not adhere and spread, but contaminating granulocytes, monocytes and thrombocytes adhered to the dish and spread. After 30 minutes the lymphocytes were removed and centrifuged at 500 rpm for 5 minutes. The lymphocytes were resuspended, adjusted to  $6 \times 10^6$  cells/ml. without further washing, and stored at 4°C until used. However, for the aspecific adherence test, one more washing was necessary to remove the trace amounts of serum or plasma present because such trace amounts of serum would prevent the adherence of cells to the surfaces. These preparations of lymphocytes were more than 90% pure (Terasaki et al., 1960).

(b) Isolation of Lymphoid Cells from Thymus and Bursa

Three to nine week old White Leghorn chickens were killed by either cervical dislocation or by injection of 0.5 ml. of Nembutal into one of the wing veins. The thymus, which consists of several lobes on both sides of the neck along the left and right carotid veins, was removed by cutting the mid center cervical region. The bursa of Fabricius was obtained from the upper portion of the cloaca. The tissues were washed with HBSS and the fatty or unwanted tissues were teased off with fine forceps in a 60 x 15 mm petri dish. The thymus or the bursa was then cut into smaller pieces and pressed through a stainless steel mesh fitted inside a 12 ml. syringe. The cells were collected and HBSS was added to the syringe for further flushing of cells. The cells collected were washed at least three times with HBSS with trace amounts of autologous plasma at 39 g or 500 rpm. The cell



suspension was adjusted to about  $1 \times 10^7$  cells/ml. and incubated in a 60 x 15 mm petri dish at 37°C for 30 minutes to remove the contaminating cells which adhered in the presence of serum. Sometimes 2 to 3 successive incubations were needed to remove all the unwanted cells. Incubation in this way also removed cellular debris. This was very important when the automatic cell counter was used. The cells were collected and washed at least 3 times with HBSS with trace amounts of autologous plasma. The cell concentration was also adjusted to  $4-6 \times 10^6$  cells/ml. These preparations contained mainly lymphocytes. The size of thymus cells were the smallest when compared to peripheral lymphocytes and the bursa cells were the biggest among the three types of lymphocytes. However, it was very difficult to prepare a cell suspension of bursa cells free of spontaneous adherence, and because of this, the background counts for bursa cells were always high.

## II. Preparation and Treatment of Alloantisera and Plasma

Antisera were prepared by conventional immunization with whole blood from birds bearing A or B antigens not borne by the recipient. The antisera were kept at -20°C until used. The precipitates present in thawed sera were removed by centrifugation and millipore filtration. Usually male recipients are preferred because the sera obtained from male birds are very clear and contain little lipid. While the female sera is almost impossible to clear up with centrifugation or to filter through a millipore filter because of high lipid levels. The specificities of all antisera were tested by hemagglutination and the sera were absorbed with erythrocytes to remove cross-reactivity. Antisera were diluted with HBSS to equivalent hemagglutinating potency prior to some tests of allofixation. The antisera and plasma used in some tests were heated at 56°C for 30 minutes to inactivate complement (Rose and





Orlans, 1962). The precipitates formed during heating were removed by filtration through a millipore AP200 filter or through tight cotton plugs. The antisera used in some tests were diluted with HBSS, dialyzed at room temperature against HBSS under negative pressure, or dialyzed overnight at 4°C against HBSS and adjusted to initial volumes with HBSS.

#### IV. Lymphocyte Adherence Test

To obtain adherence in the absence of serum proteins, 150 ul of HBSS was added to 50 ul of suspension of lymphocytes which had been washed twice in HBSS. The suspension was mixed by manual shaking and a drop was removed and counted ( $1-2 \times 10^6$  cells/ml. or 200-400 cells/0.12-0.20 mm<sup>3</sup>) in the hemocytometer under phase contrast or in later experiments with a semi-automatic cell counter (Fisher Scientific). Viable cells were highly refractile when viewed with a 10x phase contrast objective (WILD) and dead cells were not refractile. The viability of refractile cells was confirmed by their ability to exclude trypan blue (0.2% in saline). The number of refractile cells counted immediately after the addition of cell suspension to the incubating medium represented the 'zero' count and is represented in the Results as zero percent adherence. This suspension was incubated at an ambient of 25°C, except where indicated, and recounted at hourly intervals. The percent adherence was determined as the number of cells lost divided by the initial number x 100. The pH of HBSS varied from 6.0 to 6.5 and was not adjusted except where indicated. Adjustments were made with 1N or 0.1N NaOH or HCl.



For allofixation, 100  $\mu$ l of diluted antiserum was mixed with 50  $\mu$ l of fresh plasma at 0°C and then mixed with 50  $\mu$ l of lymphocyte suspension. Otherwise the test was run essentially as for adherence in the absence of serum proteins. Initially, HBSS was used as diluent for the antiserum and the pH of the mixture was  $8.3 \pm 0.2$ , but in later experiments the antiserum was diluted with HBSS supplemented with 15 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid) buffer and with pH adjusted with 1 or 0.1N NaOH to 8.5 (see Results for optimal conditions of the test). If the pH of the solution was adjusted without the presence of HEPES buffer it would tend to revert to 8.5 during prolonged incubation periods. HEPES buffer was added to stabilize the final pH of the solution (Kruse and Patterson, 1973). The pH was measured at the end of some tests as a precaution. The HBSS was made up of 8.00 g NaCl, 0.40 g KCl, 0.20 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.06 g  $\text{KH}_2\text{PO}_4$ , 0.09 g  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , 0.14 g  $\text{CaCl}_2$ , 1.00 g glucose and 0.02 g phenol red per liter (Hanks and Wallace, 1949).

## V. Cell Counting

The concentration of cells was counted with a hemocytometer or with an Autocytometer II (Fisher Scientific). For the hemocytometer count, a drop of cell suspension was drawn and transferred to the counting chamber, and the number of cells per cu. mm was counted under the phase contrast microscope (WILD) with 10x eyepieces and 10x objectives. The viable and refractile cells were counted with a cell counter, and the contaminating erythrocytes were excluded from the count. The advantages of the hemocytometer count were that I could count the type of cells I wanted to count and exclude the unwanted cells. The percent





viability of the cells was known from observation under phase and there was no difficulty with cell counting due to the presence of cell debris. The chief disadvantage of manual counting was that eyestrain limited the number of counts that could be made. In addition, the volume counted by the hemocytometer was small and the risk of a sampling error was greater.

For the automatic count by the Autocytometer II, a sample of 20  $\mu$ l was drawn from the tube containing the cell suspension with a Fisher Dilutor (Model 240) which diluted the sample automatically 1/250 with isotonic solution (Polypac). The solution was collected in a clean vial (21x 70mm Kimble glass) and then counted with the counter. Before a counting could be done, a threshold curve for the optimal operation of the machine with a particular type of cells had to be determined. This was done by counting a sample of cell suspension with the different thresholds calibrated on the machine. Two threshold calibrations are marked on the machine: RBC threshold for the red cell count and WBC threshold for the white cell count. I selected the WBC threshold for the enumeration of chicken lymphocytes. An aliquot of lymphocyte suspension was read at different threshold levels and the counts plotted against the threshold levels. Initially the count increased with the threshold and then levelled off. A threshold level on this plateau was selected for the counting of cells. I used WBC threshold "60" for the routine count of chicken lymphocyte suspension. The autocytometer would draw 0.5 ml. of sample to flush away the solution of the previous count and then draw in 0.5 ml. of new sample for counting. The number of counts was multiplied 250x to compensate



for dilution, and shown on the screen. The number of counts shown was the number per ml. of solution. The advantages of the cell counter were that I could count as many samples as I wanted to and that there was no human bias in the count. The volume of the sample counted was larger and the count was therefore, more reliable than the manual count. The drawback was that the solution had to be very clean for the counter counted any cell particles or debris or precipitates if they were over a certain size and the counter did not differentiate between viable and non-viable cells or between erythrocytes with the lymphocytes. Thus an inaccurately high count might be observed if the suspension was not properly prepared.





## Results

### I. Lymphocyte Adherence Test

Lymphocytes which have been washed twice and resuspended in HBSS adhere to glass and plastic at every pH ( Figure 1 ). Inclusion of fresh plasma inhibits this genetically aspecific adherence (Figure 1). Inclusion of antibody with fresh plasma reveals an optimal pH for allofixation between 8 and 9. This appears to be true for all the B alleles tested (Figure 2). Maximal allofixation is about the same as the aspecific adherence. The data may be somewhat misleading for cells incubated at extreme pH since 'adherence' is determined as the loss of cells from suspension. Aspecific lysis could have contributed to the loss of cells in HBSS at very low and very high pH. Aspecific lysis cannot be the explanation for the differences seen at intermediate pH since separate experiments have demonstrated recovery of more than two-thirds of the 'lost' cells.

The series of pH tests was run at 25°C. In a second series, at pH 8.5, the temperature varied from 0°C to 42°C (Figure 3). Fifty percent of the cells incubated in HBSS adhere within 1 hour even at 0°C. In contrast, allofixation is blocked at 0°C, but equals aspecific adherence at 37°C. The effect of temperature on aspecific adherence is so slight that it can be attributed to non-metabolic mechanisms, but the effect on allofixation implies the involvement of the cell's metabolism.

A series of time effect studies was done at room temperature (25°C) and pH 8.5. Figures 5 and 6 illustrate the adherence of cells with specific antisera. This could be detected at 15 minutes. Adherence



increased rapidly during the first hour of incubation. Almost all cells adhere after two hours of incubation at room temperature (25°C). A difference in the rate of adherence of homozygous and heterozygous cells could be detected at 15 minutes, but the persistence of this difference depends on the B genotype. The early onset of adherence suggests that the initial binding of antibody and alteration of the cell surface must begin almost immediately. Aspecific adherence is more rapid than the allofixation reaction (Figure 4). The adherence of cells to surfaces requires random contact between the cells and surfaces. It may not, therefore, be practical to measure adherence before 15 minutes. Since the settling of cells favors contact, but contact need not lead to adherence, the test tubes were shaken to resuspend "non-adherent" cells before each count. The frequency of shaking may have some effects on cell adherence (Figure 7). The data suggest that too many shakings at short intervals, for example at 15 minutes, may decrease the number of "adherent" cells. This is true in the sense that too many shakings might interfere with the transformation of contact to adherence though it may increase the chance of contact and the chance of cell-to-cell contact and agglutination.

The series of cell concentration effects was run at room temperature and at pH 8.5. Allofixation does not differ significantly for cell concentrations from  $0.5 \times 10^6/\text{ml}$  to  $7.0 \times 10^6/\text{ml}$  (Figure 8), but a slight increase in allofixation is observed at higher concentrations. This suggests that the cell concentration that I use for routine allofixation test, i.e.  $1.0 - 2.0 \times 10^6/\text{ml}$ , may not be optimal. However, too many cells lead to agglutination, and may complicate the use of diluted antisera.





Allofixation proceeds rapidly when the antigen is  $B_{14}$  and the antibody is anti- $B_{14}$ . The rapidity of this reaction lends itself to a test of specificity. Anti- $B_{14}$  was prepared by immunizing a  $\underline{B}^{13}/\underline{B}^{13}$  bird with  $\underline{B}^{14}/\underline{B}^{14}$  erythrocytes and by absorbing the antiserum with  $\underline{B}^1/\underline{B}^1$  and  $\underline{B}^2/\underline{B}^2$  erythrocytes. The specificity of the antiserum is based on the hemagglutination test, though as we see later this may not be a good criterion of the specificity of the antiserum for the allofixation test (see Chapter 3). Table 1 illustrates the tests of the peripheral lymphocytes of 28 birds: 4  $\underline{B}^1/\underline{B}^1$ , 6  $\underline{B}^2/\underline{B}^2$ , 4  $\underline{B}^{13}/\underline{B}^{13}$ , 6  $\underline{B}^-/\underline{B}^{14}$  and 8  $\underline{B}^{14}/\underline{B}^{14}$ . The lymphocytes of the 14 birds which lacked blood group antigen  $B_{14}$  did not adhere in significant numbers. Eighty percent or more of these lymphocytes remained in suspension in the two dilutions of antiserum tested. In contrast, the lymphocytes of the 14 birds belonging to blood group  $B_{14}$  reacted strongly. Sixty percent of the lymphocytes adhered within 1 hour and 80 percent adhered within 2 hours. The lymphocytes of one bird, a  $\underline{B}^2/\underline{B}^{14}$ , gave a relatively weak reaction which nevertheless exceeded 40 percent at 1 hour and 60 percent at 2 hours in the higher of the two concentrations of antiserum used in these tests.

The rapidity of the reaction of anti- $B_{14}$  serum with  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes makes this reaction useful in testing the contribution which fresh plasma makes to allofixation. Three series of tests were done. Untreated antiserum was used in the first series, heated antiserum in the second, and dialyzed antiserum in the third. Each series included three tests, one with fresh plasma, one with heated plasma or agamma chicken serum, and one without plasma (Figure 9). Omission of plasma



reduced the reaction with untreated antiserum from 95 to 33 percent, the reaction with heated antiserum from 80 to 5 percent, and the reaction with dialyzed antiserum from 64 to 1 percent. Although treatment of the antiserum did affect the reaction ( $F=78.71$ ,  $p<0.001$ ) the effect of omitting plasma ( $F=318.11$ ,  $p<0.001$ ) was even greater ( $F_{\text{plasma}}/F_{\text{antiserum}}=4.04$ ). Dialysis of the antiserum against HBSS (pH 7.2) made the reaction very sensitive to the presence of fresh plasma, an observation which might mean that dialysis removes a partial substitute for fresh plasma. However, transitional changes, such as pH, during dialysis might be more important than the physical removal of any constituent.

The specificities of B antisera were tested in another series of experiments which may be illustrated by the reactions of  $\underline{B}^2/\underline{E}^2$ ,  $\underline{B}^2/\underline{B}^{14}$ , and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with anti- $B_2$  and anti- $B_{14}$  sera. Anti- $B_2$  prepared by immunization of birds which lack  $B_{14}$  antigens will invariably react with  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. The converse is true for anti- $B_{14}$  prepared by immunization of birds which lack  $B_2$  antigens (Figure 10). Nevertheless, the cross-reaction will be weaker than the specific reaction and the difference will be maximal at some intermediate dilution. In this instance the difference is greatest at serum dilutions of 1/16 and 1/32. Absorption of anti- $B_{14}$  with  $\underline{B}^2/\underline{B}^2$  erythrocytes removed reactivity for  $\underline{B}^2/\underline{B}^2$  lymphocytes and reduced the reaction with  $\underline{B}^2/\underline{B}^{14}$  and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes (Figure 11). The maximal difference was shifted to serum dilutions of 1/4 and 1/8. At a dilution of 1/16 the reaction with  $\underline{B}^2/\underline{B}^{14}$  cells was eliminated, and the reaction with  $\underline{B}^{14}/\underline{B}^{14}$  cells was reduced to 30 percent adherence. At a dilution of 1/32 there was no adherence for either  $\underline{B}^2/\underline{B}^{14}$  or  $\underline{B}^{14}/\underline{B}^{14}$  cells.





When anti-B<sub>2</sub> sera are tested with a panel of  $\underline{B}^{14}/\underline{B}^{14}$ ,  $\underline{B}^2/\underline{B}^{14}$ , and  $\underline{B}^2/\underline{B}^2$  lymphocytes the reactions prove to be strikingly different from those obtained with anti-B<sub>14</sub>. At 25°C the allofixation of  $\underline{B}^2/\underline{B}^2$  cells by anti-B<sub>2</sub> is about 30 percent at 2 hours. The allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  cells by anti-B<sub>14</sub> exceeds 90 percent under the same conditions. The allofixation of  $\underline{B}^2/\underline{B}^{14}$  lymphocytes is 20 percent with anti-B<sub>2</sub> and 80 percent with anti-B<sub>14</sub> (Figure 13). The reaction with anti-B<sub>14</sub> is 3 to 4 times as great as the reaction with anti-B<sub>2</sub> one and two hours after the initial count, even when the same cells are used for both tests. If  $\underline{B}^2/\underline{B}^2$  and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes used in these tests are allowed to react with anti-A antibodies they react equally. In addition to this, the  $\underline{B}^2/\underline{B}^2$  lymphocytes are able to react strongly with the unabsorbed anti-B<sub>2</sub> prepared in a  $\underline{B}^{14}/\underline{B}^{14}$  bird (Figure 12). The different reactions with anti-B<sub>2</sub> and anti-B<sub>14</sub> sera must therefore represent some feature specific to the B antigens, their locations, their quantities, or their abilities to trigger cellular changes, rather than a difference in the adhesiveness or motility of  $\underline{B}^2/\underline{B}^2$  and  $\underline{B}^{14}/\underline{B}^{14}$  cells.

The same kinds of tests were done for four other B alleles, the  $\underline{B}^1$ ,  $\underline{B}^{13}$ ,  $\underline{B}^{15}$  and  $\underline{B}^{21}$ . The results, as illustrated in Figure 13, show that B<sub>13</sub> and B<sub>15</sub> cells adhered in the same manner as the B<sub>14</sub> did, the B<sub>21</sub> adhered as the B<sub>2</sub> cells did, and the adherence of B<sub>1</sub> cells was intermediate between these two. The reactions of B<sub>1</sub>, B<sub>13</sub>, B<sub>15</sub> homozygous and heterozygous cells are only slightly different after two hours of incubation, though the rate of adherence within the first hour of incubation may be different. The lymphocytes from donors whose erythrocytes do not agglutinate with a specific antibody do not adhere. This



further indicates the specificity of the reaction, and the similarity between the  $\underline{B}^1$ ,  $\underline{B}^{13}$ ,  $\underline{B}^{14}$ , and  $\underline{B}^{15}$  alleles in their response to allofixation. The anti- $\underline{B}_{21}$  serum used in this test is absorbed only with  $\underline{B}_2$  and  $\underline{B}_{15}$  erythrocytes because further absorption will tend to remove all reactivity for  $\underline{B}_{21}$  cells. The results of the allofixation tests for  $\underline{B}_{21}$  are very similar to tests for  $\underline{B}_2$ . However, more tests are needed to confirm the weakness of the  $\underline{B}^2$  and  $\underline{B}^{21}$  alleles in this system because the weak reaction of  $\underline{B}_2$  and  $\underline{B}_{21}$  cells with their respective antibodies may be due to low levels of allofixing antibody (see Chapter 3). It may not be due to a quantitative or qualitative difference of the antigens or their distributions on the lymphocyte surface. This can only be proved by reaction with isotope-labelled antibodies. This type of experiment is in progress.

When anti- $\underline{A}_2$  and anti- $\underline{A}_6$  sera are tested with a panel of  $\underline{A}^2/\underline{A}^2$ ,  $\underline{A}^2/\underline{A}^6$ , and  $\underline{A}^6/\underline{A}^6$  lymphocytes, the results are startling. These tests were initiated as a control of the B reaction because A antigens have never been detected on chicken lymphocytes and are not known to influence graft rejection. Allofixation by anti-A sera is stronger and more rapid than the specific reaction mediated by anti- $\underline{B}_2$  and  $\underline{B}_{21}$  although it is not as strong as the reaction mediated by anti- $\underline{B}_{13}$ , and anti- $\underline{B}_{14}$  and anti- $\underline{B}_{15}$  and probably anti- $\underline{B}_1$ . The  $\underline{A}_2$  and  $\underline{A}_6$  reactions are indistinguishable except for their specificities. When  $\underline{A}_2$  and  $\underline{A}_6$  reactions are analysed the percent adherence is seen to be a linear function of the number of  $\underline{A}^2$  and  $\underline{A}^6$  alleles borne by the donor bird (p for linear regression <0.001; p for deviation from linearity >0.05). This is recognizable as an exact expression of gene dosage (Longenecker et al., 1972) and is most easily interpreted as meaning that the  $\underline{A}$  heterozygotes





have exactly one-half as much  $A_2$  (or  $A_6$ ) antigen as the  $\underline{A}^2/\underline{A}^2$  (or  $\underline{A}^6/\underline{A}^6$ ) homozygotes. The A reaction might possibly be due to reaction with A antigen in the autologous plasma in which these tests were done. This possibility was eliminated by tests in which the cells and the fresh plasma came from different  $\underline{A}$  genotypes, i.e.,  $\underline{A}^2/\underline{A}^2$  cells plus  $\underline{A}^6/\underline{A}^6$  plasma and  $\underline{A}^6/\underline{A}^6$  cells plus  $\underline{A}^2/\underline{A}^2$  plasma. The substitution of another plasma for autologous plasma had no effect. Allofixation with anti-A sera is determined by the genotype of the donor of the lymphocytes, and not that of the donor of the plasma. This does not, of course, exclude the possibility that A antigens are absorbed onto the lymphocytes in vivo.

Adherent cells have been recovered by treatment of the surfaces with 0.25% trypsin solution or by vigorous agitation. The cells appear normal, but normal appearance does not prove normal function and not all of the cells can be recovered. It would in any case be difficult to prove that all adherent lymphocytes retain all of their normal functions. To test normal function, I resorted to the graft-versus-host (GVH) competence of adherent cells. Although only 1 of every 1,000 to 10,000 lymphocytes can normally be shown to have GVH reactivity it is possible to test the effect of immunoadherence of this small population of lymphocytes with precision. A circular coverslip was placed in contact with an HBSS suspension of a known number of lymphocytes. Another coverslip was placed in contact with a similar suspension which differed only by the prior addition of fresh plasma. A third coverslip was placed in contact with a similar suspension which differed only by the prior addition of fresh plasma and B- specific





antibody. Fourteen sets of coverslips were used. After incubation for 1 hour at 25°C the coverslips were washed vigorously and repeatedly in HBSS, inverted, and placed on the chorioallantoic membranes (CAM) of chick embryos bearing B antigens to which the lymphocytes should react. The cells left in suspension when the coverslips were removed were collected and used to test other chick embryos. The number of chorioallantoic pocks expected, based on prior tests, was 1600. The cells which adhered to coverslips when suspended in HBSS accounted for 1404 pocks and the cells which did not adhere accounted for 2 pocks (Table 2). The cells which adhered to coverslips when suspended in HBSS plus fresh plasma accounted for 8 pocks and the cells which did not adhere accounted for 775 pocks. The cells which adhered to coverslips in HBSS plus fresh plasma plus B antibody accounted for 1465 pocks and the cells which did not adhere accounted for 181 pocks. It is clear that GVH competent lymphocytes do not suffer an irreversible loss of GVH competence as a consequence of adherence. Since GVH pocks are internal to the CAM the experiment also indicates that the lymphocytes can detach, migrate, and respond to histocompatibility antigens.

## II. Distribution of A and B Alloantigens on Peripheral Lymphocytes, Bursa and Thymus Cells

In the mouse at least six distinct systems of lymphocyte surface antigens are recognized by the cytotoxic effects of specific antisera (Boyse et al., 1968). In the chicken, bursa- and thymus-specific antigens have been identified by immune adherence (Forget et al., 1970), by  $\text{Cr}^{51}$  release (Ivanyi and Lydyard, 1972), and by immunofluorescence (Hudson and Roitt, 1973). Mammalian antisera were used for these tests.



The distribution of antigens among the lymphoid organs is known to differ. The H-2 alloantigens of mice are poorly represented on the thymus cells (Raff, 1971), TL antigens (Old et al., 1963) are restricted to leukemic cells and thymus cells of some strains, and theta antigens are only found on the thymus cells or the thymus derived cells and the nervous tissue (Raff, 1969). The present study was undertaken to study the expression of A and B alloantigens on the thymus and bursa cells of the chicken, and to compare this with the expression of these antigens on the peripheral lymphocytes.

Lymphocytes of peripheral blood, thymus and bursa of chickens 3 to 9 weeks old were tested for B antigens. Figure 14 illustrates the expression of the B<sub>14</sub> alloantigens on the peripheral lymphocytes (P), the thymocytes (T) and the bursa cells (B) from 3 to 9 week old chickens. The results indicate that the B<sub>14</sub> alloantigen is fully expressed on the P cells at the age of 3 weeks and no further development of this antigen is observed from 3 to 9 weeks after hatching. However, B<sub>14</sub> antigens are not equally distributed on different lymphoid cells. This shows similarities to the H-2 distribution in the mouse. B<sub>14</sub> antigens are not detected on the thymus cells, are poorly expressed on the bursa cells, and are strongly expressed on the peripheral blood lymphocytes (Figure 15). Seventy-five percent of the P cells, 30 percent of the B cells and only 10 percent of the T cells were fixed by alloantibody. This result is in accord with the finding in the H-2 system of mice where H-2 antigens are poorly represented on thymus cells (Raff, 1971). The number of adherent cells in the thymus appears to increase slightly with increase of age. This may represent the presence of "P" cells in the older thymus.





Peripheral lymphocytes, thymus cells and bursa cells of  $\underline{A}_6/\underline{A}_6$  chickens reacted with anti- $A_6$  (Figure 16). Seventy to eighty percent of the cells were fixed. Relatively few P and T cells adhered when suspended in autologous plasma but 20 percent B cells adhered. This high background for B cells is not interpretable at this time. An antiserum specific for  $A_2$  caused P and T cells to adhere (Figure 16). Bursa cells were not affected. This suggests that this antiserum contained tissue-specific antibodies for thymus and the peripheral lymphocytes. This is my only evidence for an antigen analogous to the theta antigen of the mouse (Reif and Allen, 1964). Thymus-specific and bursa-specific antigens have been reported by others (Forget et al., 1970; Hudson and Roitt, 1973; Ivanyi and Lydyard, 1972). McDermid (1968) and Thein and Schmid (1968) also reported alloagglutination of lymphocytes independent of hemagglutination.



## Discussion

The present work began with a consideration of Terasaki's comment that chicken lymphocytes treated with alloimmune sera remain stationary although the formation and withdrawal of small pseudopods continues (Terasaki et al., 1960). The immobility was attributed to the failure to form large pseudopods and to possible adherence to surfaces. This suggested to me the possibility that the immobilization of lymphocytes at surfaces may be used in an immunospecific manner. My first attempts to do this, by exposure of lymphocytes to surfaces to which alloantibodies had been covalently bonded, encountered serious difficulties. In the course of these experiments it became evident that rewashed lymphocytes do not remain in suspension if placed in standard solutions of salts, i.e., HBSS, HBSS minus calcium and magnesium, Seligmann's (Graber et al., 1955), or sodium chloride. The loss of lymphocytes is so rapid even at 0°C as to make lysis an improbable explanation. The 'lost' lymphocytes are found firmly adherent to untreated glass and plastic surfaces. When removed from these by vigorous agitation or treatment with trypsin, these lymphocytes appear normal and active and readhere without difficulty. Most small lymphocytes adhere within the first hour, even at 0°C. There appear to be no differences among chickens with respect to this kind of adherence which I term "aspecific adherence."

In contrast to the rapid adherence of rewashed lymphocytes suspended in HBSS, rewashed lymphocytes suspended in HBSS containing plasma, serum, or serum proteins remain in suspension for many hours





(as do once-washed lymphocytes). The addition of one part fresh plasma to 1,000 parts HBSS inhibits the adherence of rewashed lymphocytes. Higher concentrations of fresh plasma suppress adherence completely. If, in addition to fresh plasma, the HBSS contains A or B alloantibodies lymphocytes of the proper genotypes will adhere. This allofixation, in contrast to the aspecific adherence which occurs in protein-free suspensions, is enhanced rather than inhibited by fresh plasma. It is greater at higher concentrations of plasma. A 1-in-4 or 1-in-8 mixture of fresh plasma in HBSS is convenient for tests of allofixation. Higher concentrations are impractical and lower concentrations have less buffering capacity.

The importance of fresh plasma for allofixation is most evident when HBSS-dialyzed antisera are used. Dialyzed antiserum by itself does not fix lymphocytes to surfaces, but the addition of fresh plasma initiates rapid allofixation. The effect of omitting fresh plasma is somewhat less in tests with fresh and heated antisera. The fact that treatments which inactivate complement abolish allofixation suggests that the synergistic component in fresh plasma may be complement, but I have made no serious attempts to test this.

The specificity of plasma-enhanced adherence has been demonstrated for 6 B alleles, B<sup>1</sup>, B<sup>2</sup>, B<sup>13</sup>, B<sup>14</sup>, B<sup>15</sup>, and B<sup>21</sup>, and for two A alleles, A<sup>2</sup> and A<sup>6</sup>. The greatest useful dilution of specific antiserum is about one-half the hemagglutination titer of hyperimmune sera. Lower dilutions of antiserum are not useful because of prozone effects and the occurrence of lymphoagglutination. Thus, my evidence that allofixation is immunologically and genetically specific is based on



the levels and rates of adherence at intermediate dilutions of antisera. At these dilutions it is possible to discriminate  $\underline{A}^2$ ,  $\underline{A}^6$ , and  $\underline{B}^2$  homozygous from heterozygous chickens, with anti- $A_2$ , anti- $A_6$ , or anti- $B_2$  respectively.  $\underline{B}^1$ ,  $\underline{B}^{13}$ ,  $\underline{B}^{14}$ , and  $\underline{B}^{15}$  homozygotes and heterozygotes can, of course, be discriminated by reactions which detect the alternate allele of the heterozygote.

Allofixation depends on three components: the proper A or B antigens, antibodies to these antigens, and unknown components which are particularly abundant in fresh plasma. The manner and sequence in which these interact is unknown. My attempts to adsorb antibodies to surfaces and to use these coated surfaces to bind lymphocytes have not succeeded. All of my data are consistent with the possibility that the antibodies combine with A and B antigens of the lymphocyte prior to the adherence of the lymphocytes to the surfaces. In this respect the reaction resembles established methods in which cells react with heteroantibodies and then adhere to or bind other cells, e.g., chicken embryo fibroblasts coated with mammalian anti-chicken fibroblast-sera will bind human erythrocytes (Schwartz et al., 1969). The term "allofixation" has been introduced for several reasons inherent in the method, but its principal merit may be in distinguishing it from other forms of immunoadherence. Allofixation should not be confused with the fetuin-mediated adherence of phagocytes or the usual adherence to surfaces of neutrophils, monocytes, and thrombocytes. Adhering lymphocytes share with other cells one important feature--the lymphocytes adhere singly and separately in the concentrations used and not, as might be expected, as clumps. Allofixation does not cause the cells to flatten--they keep their globular shape.





## Conclusion

I have described a new technique for the detection of allo-antigens based on the immobilization of cells at surfaces. The technique is probably more sensitive than immunofluorescence and lymphoagglutination as indicated by the ability of allofixation to detect certain antigens that were not detected previously by those methods. It is recommended that allofixation be done at pH 8.5 at either room temperature (25°C) for 2 hours or at 37°C for 1 hour. Under these conditions specific adherence is maximal and the reaction may be regarded as complete.

The technique requires the addition of certain heat sensitive factors present in fresh chicken plasma. The shaking of the tube before counting the cells in suspension is necessary in order to resuspend cells which have settled aspecifically, but too many shakings may reduce the specific adherence. Shaking may interfere with the test by causing lymphoagglutination.

The specificity, high yield, and non-lytic characteristics of allofixation may prove to be very useful for general application in the separation of different functional groups of cells from heterogenous populations of lymphocytes. The present technique may be useful for the isolation of cells bearing specific receptors for phyto mitogens, Concanavalin A and Phytohemagglutinin; for anti-T, anti-B, anti-allotype antiserum; for hormones, and for components of complement.





## Chapter 2

### Mechanism of Cell Adherence

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## Introduction

The adherence of healthy cells to surfaces, whether mediated by the removal of plasma protein or the addition of alloantibody, must involve subtle changes in the cell surface. The nature of these remains unknown, but their ultimate recognition is likely to depend on the degree to which adherence can be related to experiments with non-lymphoid cells. Cell membranes may be altered with variable selectivity by proteolytic enzymes or chemical agents (Burger and Goldberg, 1967; Inbar and Sachs, 1969; Moscona, 1971; Springer, 1963). Proteolysis can have profound effects on the expression of antigens and can reveal hidden macromolecules or remove expressed molecules (Bhandari and Singel, 1973; Chapel and Welsh, 1972; Gibofsky and Terasaki, 1972; Mittal et al., 1969; Schwartz and Nathenson, 1971; Turner et al., 1972). Neuraminidase can alter cell behaviour or adhesiveness and this is attributed to the removal of terminal sialyl residues and the consequent alteration of the electro-potential of the cell surface (Kassulke et al., 1971; Lichtman and Weed, 1970; Sanford, 1967; Shimada and Nathenson, 1971; Weiss, 1963). The importance of terminal residues is equally evident from studies of the ABO antigens of the human erythrocyte surface. Whether terminal and expressed, or hidden, the structural and chemical components involved in adherence may belong to the extra-membranous coating or glycocalyx of the cell. Some components, presumably located in the membrane itself will, if reacted with multi- or polyvalent binding agents, aggregate and eventually form a cap at one pole. It is feasible to apply the multivarious methods which underlie these observations to the chicken lymphocyte.



## Materials and Methods

### I. Chemicals and Biochemicals

The chemicals used were D-glucosamine HCl, D-galactosamine HCl, D-mannosamine HCl, N-acetyl-glucosamine, N-acetyl-galactosamine, N-acetyl-mannosamine, cyclohexylamine, hydrocortisone-21-sodium-succinate, chloramphenicol, 2,4 dinitrophenol, 2-deoxy-D-glucose (Sigma), L-fucose, D-galactose, D-mannose and bovine serum albumin (crystalline) (Nutritional Biochemicals), sodium borate, sodium periodate, sodium cyanide, sodium citrate, disodium ethylenediaminetetraacetate (EDTA), hexamethylene amine, sodium chloride, magnesium chloride, calcium chloride, sucrose and gelatin (Fisher Scientific), iodoacetamide (K & K Laboratories), heparin (Connaught Medical Research Laboratories), vinblastine sulfate (Velbe) (Eli Lilly) and agar and trypsin (1:250) (Difco).

### II. Lymphocyte Suspensions

Lymphocyte Suspensions were prepared as described previously in Chapter 1.

Lymphocytes were x-irradiated in HBSS, with trace amounts of plasma. The dosages were 500R (25R/min. for 20 minutes); 1000R (200R/min. for 5 minutes); and 5000R (200R/min. for 25 minutes). For the aspecific and specific adherence tests, non-irradiated lymphocytes from the same preparation were used as the control.

Lymphocytes were trypsinized by incubation in 0.25% trypsin in HBSS (pH 7.5) at 37°C for 10 minutes, in a petri dish (60 x 15 mm). They were washed twice and resuspended in HBSS. To prevent aspecific





adherence of cells, a trace amount of plasma was added. The cell concentration was adjusted with HBSS to 4 to  $6 \times 10^6$  cells/ml. and the non-treated lymphocytes of the same preparations were used as controls.

### III. Substrates

Pyrex and Falcon plastic test tubes were used. All tubes were 12x75 mm. Plastic tubes were coated by adding 1 ml. of 0.5% gelatin, agar or BSA solution in HBSS to each tube and were allowed to stand in the refrigerator overnight. The tubes were rinsed with distilled water 3 times and air-dried before use.

Antiserum treated dishes were prepared by adding 2 ml. of diluted antiserum to each dish (35x10 mm). The dish was incubated with the antiserum at room temperature for 2 hours. The dish was then washed or rinsed with HBSS 2 to 3 times and used directly for the test. Cells suspended in 25% fresh plasma (occasionally 12.5%) were added to the dish. The antiserum treated dish was further incubated with 0.85% saline, 0.25% pepsin or papain solution in some tests. Both the reagents were obtained from Sigma. The dish was then washed twice with HBSS before the addition of cells. Cell adherence was observed under the phase microscope and the loss of cells was counted by means of a cell counter.

### IV. Inhibition Assay

Simple sugars, amines, amino sugars, anticoagulants, inhibitors of metabolism and macromolecular synthesis, hydrocortisone, sodium borate, sodium periodate, and vinblastine were tested as possible



inhibitors of allofixation and aspecific adherence. Fifty  $\mu$ l of cell suspension was added to a tube containing a mixture of 25  $\mu$ l autologous plasma, 25  $\mu$ l antiserum and 100  $\mu$ l HBSS with or without supplement of 15mM HEPES buffer. The pH was adjusted to 8.5 with 0.1N NaOH or HCl. The 100  $\mu$ l HBSS contained 2 times the concentration of the reagents or inhibitors used in the test. The pH of the mixture was usually not adjusted with NaOH or HCl if the final pH was not affected by the addition of the reagents. Otherwise the pH of the mixtures was adjusted to 8.0 or 8.5. The aspecific adherence test was performed by adding 50  $\mu$ l of twice washed serum-free lymphocyte suspension to a mixture of 50  $\mu$ l HBSS and 100  $\mu$ l HBSS containing 2 times final concentrations of the reagents. The pH of the solution was not adjusted unless the pH of the mixture was either too high or too low. In such cases the pH was adjusted to 7.2 with 0.1N NaOH or HCl. In some of the tests, known amounts of divalent cations were added to the mixture before the test.





## Results

### I. Chemical Nature of B Alloantigens

The chemical nature of B alloantigens was studied by the inhibition method. Figure 17 illustrates the effect of three simple sugars, L-fucose, D-galactose and D-mannose on the adherence of lymphocytes induced by alloantibodies. Four concentrations of sugars (0.25, 0.50, 0.75 and 1.00%) were tested and the results do not indicate that these sugars inhibit allofixation. Aspecific adherence was not inhibited by D-galactose or D-mannose (Figure 18). The amides and amines, N-acetyl-galactosamine, N-acetyl-mannosamine, N-acetyl-glucosamine, cyclohexylamine and hexaethylenamine were tested at concentrations of 0.25, 0.50 and 0.75%. The results presented in Figure 19 did not show inhibition by any of the amines tested and enhancement of adherence was observed with some amines. This suggests that simple sugars and their amines are probably not the antigenic determinants of the B alloantigens. However, glucosamine-HCl, an amino sugar, was found to inhibit allofixation but not the aspecific adherence (Figure 20). I believe that this inhibition is an artifact due to the marked pH changes after the addition of glucosamine-HCl. The adjustment of pH with 0.1N NaOH to pH 8.0 resulted in a reduction of the inhibition by glucosamine-HCl. The bar diagram of Figure 20 illustrates the results of the effects of the 3 amino sugars (glucosamine-HCl, galactosamine-HCl and mannosamine-HCl) on allofixation. At a concentration of 0.5%, only a slight reduction of cell adherence is detected and it is very different from





the complete inhibition of cell adherence without proper adjustment of the pH. This slight inhibition by amino sugars may be due to the changes of molarity of the solution. These failures may indicate that determinants of B alloantigens which are effective in allofixation are not identical with any of the carbohydrates tested.

This interpretation of the non-carbohydrate nature of B alloantigens is strengthened by the findings with sodium periodate and sodium borate. These two reagents modify the structure of some carbohydrates (Glaeser et al., 1968; Moscona, 1962; Zatz et al., 1972; Zittle, 1951). Figure 21 and 22 show that  $1.0 \times 10^{-3}$  to  $1.0 \times 10^{-2}$  M sodium periodate and  $1.0 \times 10^{-2}$  to  $4.0 \times 10^{-2}$  M sodium borate do not interfere with allofixation or aspecific adherence (Figures 21 and 22). This suggests that oxidation of the vicinal hydroxyls of some carbohydrates, to aldehydes, does not alter allofixation.

The removal of surface antigens can be accomplished by some proteolytic enzymes (Natenson and Davies, 1966; Turner et al., 1972), and some hidden antigens may be revealed by short treatment of cells with enzymes (Gibofsky and Terasaki, 1972; Kassulke et al., 1971; Mittal et al., 1969). The effect of trypsin, a proteolytic enzyme, on allofixation tests is illustrated in Figure 24. The results show that cells treated with trypsin adhere almost as effectively as untreated lymphocytes. The optimal pH for adherence of the trypsinized and untreated cells is still the same, i.e., between pH 8.0 and 9.0. However, a slight difference in the allofixation reaction was found between cells bearing B<sub>2</sub>, B<sub>14</sub> or B<sub>15</sub> alloantigens. How real is this difference is not known. The enzyme does not seem to affect the cell



adherence of  $B_{14}$  cells (Figure 24a), but the sensitivity is increased for  $B_{15}$  cells (Figure 24b) and a slight decrease in sensitivity is noted with  $B_2$  cells (Figure 24c). The significance of these changes is unknown and although they may be interpreted as the destruction of some  $B_2$  antigens and unmasking of  $B_{15}$  antigens, the inconsistency of the results makes interpretation difficult.

## II. Maintenance and Alteration of Topography of Membrane Structures

The cell membrane can be stabilized by the drug hydrocortisone (Dingle et al., 1967; Fell et al., 1966; Fell and Weiss, 1965). Lysis of cells by cytotoxic antibody and complement was inhibited by high concentrations of hydrocortisone (Jennings, 1966; Jennings and Taylor, 1964). However, several investigators have reported that hydrocortisone has no effect on immune lysis in vitro (Irvine, 1960; Latta, 1957; Weiss and Dingle, 1964). The discrepancy between these two groups of reports is probably due to the concentration of hydrocortisone used. The effect of hydrocortisone on allofixation was tested at 0.01 to 1.00 mg/ml. Figure 23 shows that hydrocortisone does not appear to inhibit the cell adherence induced by the alloantibody even at high concentration of the drug. Aspecific adherence was inhibited by 0.5 to 1.0 mg/ml. of hydrocortisone in association with the death of cells. It appears that a high concentration of hydrocortisone is toxic to the cells suspended in protein-free HBSS. Allofixation is not inhibited by 5 to 15 mM 2-deoxy-D-glucose (Figure 25) or 1 to 5 mM iodoacetamide (Figure 26). Since these two agents are inhibitors of glucose metabolism by the glycolytic pathway, the results suggest that normal





metabolism is not essential for allofixation. This was substantiated by tests of inhibitors of oxidative phosphorylation. Figure 27 shows that 1 to 5 mM sodium cyanide and Figure 28 shows that 0.25 to 1.00 mM 2,4 dinitrophenol have no effect on the allofixation reactions. The lack of any energy requirement for the allofixation reaction suggests that a topographic change of the cell membrane may not be an energy-dependent process and the binding of antibody to the antigens on the cell membrane is enough to produce some non-energy dependent alterations or rearrangements of cell membrane necessary for the adherence. The fact that aspecific adherence is inhibited by iodoacetamide (Figure 26) and 2,4 dinitrophenol (Figure 28), but not by 2-deoxy-D-glucose (Figure 25) and sodium cyanide (Figure 27) indicates that some type of energy-dependent process is needed for such cell adherence.

The formation and maintenance of microtubules inside the cells is required for the adhesion and spreading of polymorphonuclear leukocytes (PMN), monocytes and most cultured cells (Allison et al., 1971; Wessells et al., 1971; Zigmond and Hirsch, 1972). Vinblastine which combines with the protein subunits of microtubules (Marantz et al., 1968; Weissenberg et al., 1968) inhibits the formation of microtubules was tested. Figure 29 indicates that 1 to 100 ug/ml. of vinblastine had no effect on allofixation or aspecific adherence. It is probably the process of cell spreading which requires microtubules and the lymphocytes do not spread during allofixation or aspecific adherence.

The adhesion of many cultured cells to their substrates requires the synthesis of some surface material to facilitate adhesion





or the spreading of cells (Rosenberg, 1962; Takahashi and Okada, 1972). The adhesion of these cells is inhibited by some drugs which inhibit DNA, RNA or protein synthesis. DNA synthesis is inhibited by exposing cells to X-irradiation. Figure 30 shows that X-irradiation at 500, 1,000 and 5,000 roentgens does not affect the allofixation reaction. The inhibition of mitochondrial protein synthesis was tested with chloramphenicol at 250 to 1000 ug/ml. Figure 31 indicates that mitochondrial protein synthesis is not required for allofixation and aspecific adherence. My data appear to be in accord with the report (Frye and Edidin, 1970) that a reorganization of membrane is possible even in the presence of some protein and nucleic acid inhibitors.

### III. Roles of Divalent Cations

The effect of 3 anticoagulants was examined. Figure 32 illustrates that 5 to 50 units/ml. heparin and 10 to 30 mM sodium citrate have no effect on the adherence of cells induced by alloantibodies, but 10 to 30 mM disodium ethylenediaminetetraacetate (EDTA) completely inhibits the fixation of cells. The inhibition of allofixation by EDTA suggests the importance of divalent cations. Figure 33 indicates that 3mM EDTA is strong enough to inhibit the allofixation reaction. The cells appear abnormal when viewed with the phase contrast microscope. The cells do not appear to be lysed by these concentrations of EDTA but the refractility of the cells is lost. The non-refractile cells are seen after two hours of incubation. The inhibition by EDTA may be through inhibition of the binding of divalent cations with complement or it may be toxic to the cells because of its chelating nature. The



inhibition by EDTA can be reversed by adding divalent cations. Figure 34 shows that both calcium and magnesium ions reverse the inhibition of allofixation, but the inhibition is not reversed by the addition of sodium ions. Figure 35 indicates that 1 and 2 mM of calcium, magnesium or manganese ions do not reverse the inhibition of 5 mM EDTA, but 3 mM or more of the divalent cations are able to do so. The aspecific adherence was not inhibited by 1, 3 or 5 mM of EDTA. This indicates that the adherence of cells to surfaces in the serum-free medium does not require the divalent cations. The same observation has been reported by many investigators on the aspecific adherence of cells to surfaces in the serum-free medium (Berwick and Coman, 1962; Easty et al., 1960; Nordling et al., 1965; Rosenberg, 1960; Takeichi, 1971; Taylor, 1961).

#### IV. Roles of Surface Properties of Substrates

The surface properties of the substrates are important for the adherence of cells (Nordlings et al., 1965). By coating a substrate with serum (Taylor, 1961) or chemicals such as fatty acid (Rosenberg, 1962, 1963) or metal (Carter, 1965), the adherence of cells is greatly affected. The deposition of extracellular materials on the substrates by the cultures also alters the properties of the substrates, and the adherence of cells is affected (Rosenberg, 1960; Weiss, 1961). The effect of surface properties on the aspecific adherence and allofixation were studied with the uncoated glass and plastic and with plastic coated with protein or agar. Aspecific adherence is not affected by the substitution of glass for plastic, but allofixation is (Figure 36).





Allofixation on plastic or pyrex is superior to allofixation on some other glasses. The difference is probably due to the chemical composition of the glasses (Nordling et al., 1965; Taylor, 1961). Figure 37 shows that the coating of plastic with gelatin, bovine serum albumin (BSA) or agar, greatly reduces the fixation of cells to these substrates. Both the aspecific and specific adherence are affected. This suggests that the lymphocyte can discriminate uncoated from coated surfaces; perhaps due to the difference in electropotential.

The pH dependence of adherence to uncoated plastic and plastic coated with gelatin, BSA or agar was tested since this should differ if coating acts by altering the surface charge. HEPES buffer was added and pH adjusted with 0.1N NaOH or HCl. Figure 38 shows that aspecific adherence to uncoated plastic and BSA-coated plastic is largely independent of pH. Adherence is slightly increased with the increase of pH for the agar-coated plastic, and the reverse is true for the gelatin-coated plastic. However, the maximal adherence does not equal that for uncoated plastic. The increase in adherence, with decreased pH, on gelatin-coated plastic may be due to the reduction of net negative charges of the substrate as the pH decreases (Dan, 1947). On the contrary, the increase in cell adherence, with increased pH, for agar-coated plastic cannot be explained with the changes in negative charges. The reduction of net negative charges cannot explain the increase in allofixation from pH 6 to 10. The optimal pH for allofixation on both the coated or uncoated surfaces is the same, and it is usually found between pH 8 and 9. No adherence by alloantibodies is found at pH 7 or lower than this. If the negative charges do play a part in the fixation





of cells, the increase in pH from 6 to 10 should decrease the cell adherence. This appears not to be true.

The immobilization of cells to surfaces can be facilitated by the binding of a variety of molecules such as lectins, antibodies or antigens to surfaces with covalent binding agents (Edelman *et al.*, 1971; Truffa-Bachi and Wafsy, 1970; Wigzell and Andersson, 1969; Wigzell and Mäkellä, 1970). However, the removal of bound cells from the surfaces of fibers, beads, glass or plastic by a competitive inhibitor was difficult to achieve due to the limited number of known inhibitors (Edelman *et al.*, 1971; Wigzell and Andersson, 1969). Cells, antigens or antibodies have been separated specifically with various degrees of success by eluting with the specific inhibitors. On the other hand, the adsorption of a variety of molecules such as serum, purified proteins and metals to surfaces have also been described (Carter, 1965; Nordling *et al.*, 1965; Rosenberg, 1962, 1963; Takeichi, 1971; Taylor, 1961; Weiss, 1959). My early attempts to bind antibodies covalently with glutaraldehyde to collagenous surfaces were unsuccessful. However, the treatment of plastic culture dishes with high titered hemagglutinating antisera was found to facilitate specific adherence of erythrocytes to these antibody adsorbed surfaces (Table 3). Only an occasional lymphocyte adheres to these antibody-coated surfaces.  $\underline{B}_2^2/\underline{B}_2^2$  erythrocytes adhere to anti- $\underline{B}_2$  treated dishes but the  $\underline{B}_{14}^{14}/\underline{B}_{14}^{14}$  erythrocytes do not, and the reverse is true for anti- $\underline{B}_{14}$  treated dishes. Neither  $\underline{B}_2^2/\underline{B}_2^2$  or  $\underline{B}_{14}^{14}/\underline{B}_{14}^{14}$  lymphocytes are found to adhere to these surfaces. This suggests that the adsorption of antibodies to surfaces does occur. The antibodies appear to be weakly or reversibly bound, because the adherence of erythrocytes is greatly reduced by pre-incubation of the



treated surfaces with 0.85% saline at room temperature for one hour. Papain and pepsin (0.25%) treatment of the surfaces also reduces the adherence of erythrocytes to the coated surfaces although it is not clear that this reduction represents proteolysis of antibody. The adherence of erythrocytes to the antibody coated surface does not appear to require fresh serum factors; the erythrocytes adhere in both agamma chicken serum and in fresh chicken plasma. This suggests that the allofixation of erythrocytes differs from the allofixation of lymphocytes, which must be suspended in fresh chicken plasma or serum and which do not require pre-absorption of antibody to the surface.





## Discussion

It is evident that inhibition assay with monosaccharides as a means of elucidating the structure of antigenic determinants of B allo-antigens on lymphocytes was uninformative. Of all the monosaccharides, none of those tested were found to inhibit the specific fixation of lymphocytes. The simple inhibition by monosaccharides in the human ABO system was useful in elucidating their chemical nature (Watkins, 1966). The H-2, HLA and B systems have been reported to be associated with membrane lipoproteins or glycoproteins (Davidenas, 1970; Reisfeld and Kahan, 1970). Treatment of the cells with sodium borate, sodium periodate, or trypsin did not alter allofixation, and failed thereby to provide evidence for the chemical character of the B antigens,

I believe that there are at least three different mechanisms represented by allofixation of lymphocytes, aspecific adherence of lymphocytes, and the spreading adherence of PMN, phagocytes, monocytes and cells in culture. The rapid adherence of lymphocytes to surfaces in serum-free media does not require the presence of divalent cations, and the adherence occurs too rapidly to assume the synthesis of any interfacial material between cell and surfaces as a prerequisite for the initiation of cell adherence. It is possible that physico-chemical forces may draw the cell surface to the substrate surface. There have been several reports concerning the production of extracellular materials in the facilitation of cell adherence (Rosenberg, 1960; Weiss, 1961). The partial or complete inhibition of cell adherence by adsorption of materials to the plastic suggests that adherence is dependent upon the physico-chemical properties of the substrates.



Though it is true that cells require divalent cations for cell-to-cell adherence, the cells can attach to a glass or plastic surface in the absence of these cations, as long as a serum-free medium is used (Berwick and Coman, 1962; Easty et al., 1960; Rosenberg, 1960; Takeichi, 1971; Taylor, 1961). The aspecific adherence of lymphocytes in the serum-free medium is in accord with these reports. However, the specific adherence induced by alloantibodies was completely blocked by 3 mM of chelating agent, EDTA. This inhibition is associated with the alteration of surface properties of the cells which are non-refractile and appear to be dead in that small pseudopod protrusion has ceased. This inhibition of allofixation can be prevented by the addition of a suitable amount of divalent cations suggesting that the integrity of the cell membrane requires the divalent cations. Several theories have been proposed to explain the function of divalent cations in cell adherence. Curtin (1960, 1962, 1966) suggested that the action of divalent cations is to suppress the negative charge on cell surfaces, thereby reducing the electrostatic repulsive forces between cells. If the divalent cations act solely in such a way, the cell adherence should be decreased with increasing surface negative charges on cells and substrates due to rise of pH. This appears to be unlikely since the adherence of cells in the absence of serum was not affected by the change of pH and the specific adherence induced by alloantibodies increased with the increase of pH.

The surface of glass and plastic are negatively charged as are the cell surfaces. Repulsion should be an important barrier to adherence. To overcome this repulsion it may be necessary for a cell





to alter its shape. A close relation between adherence and morphology was observed. The adherent cells appeared to be more spherical than the original irregular and elongated shapes, and the adherent cells were found to be actively protruding pseudopods from all parts of the cells in contrast to the protrusion of pseudopods from only the anterior portion of the unattached cells. The undulation of cell surfaces or protrusion of pseudopods seems to be an important step in adherence to negatively charged substrates because the potential energy barrier preventing cell contact can be reduced by reducing the radius of curvature of mutually approaching cell processes (Bangham and Pethica, 1961). In fact, cells are observed to adhere to the substrate by processes on the cell surface facing the substrate (Ambrose, 1961; Curtis, 1964; Eguchi and Okada, 1971; Taylor and Robbins, 1963), and Lesseps (1963) found that cell contact is actually initiated at the crests of the undulating surfaces of cells.

The aspecific adherence appears to be an energy dependent process since both iodoacetamide and 2,4 dinitrophenol inhibit the aspecific adherence, but sodium cyanide and 2-deoxy-D-glucose do not. The inhibition of aspecific adherence by hydrocortisone suggests that membrane fluidity is a factor in adherence.

On the contrary, the specific adherence induced by alloantibodies is not affected by inhibitors of metabolism or nucleic acid synthesis, or hydrocortisone. This indicates that allofixation may not be dependent on metabolism processes. The polarized concentrations or "cap formation" of immunoglobulin determinants on the cell surface of Ig--carrying lymphocytes or after antigen-antibody and lectin-receptor





interaction on lymphocytes were found to be an energy requiring process (Sällström and Alm, 1972; Taylor et al., 1971), though the actual binding of lectins or antigens to the cell surface does not require active metabolism. It is thus evident that "cap formation" which is an energy dependent process is not required for the adherence of cells induced by alloantibodies. The simple interactions of cell surface antigens with their antibodies are able to trigger a series of changes which probably alter or modify the normal morphology of the cells through some type of modification of the cell membrane. This alteration of cell membrane probably increases the contact surface of the cell to the substrate, and facilitates the adherence of cells. Bangham and Pethica (1961) suggested that the potential energy barrier preventing cell contact can be reduced by reducing the radius of curvature of mutually approaching cell processes. The "cap formation" is probably a way to concentrate a foreign material for interiorization or pinocytosis in the cells. This process may be associated with the sensitization of the cells.

The moving of antigen-antibody aggregate or lectin-receptor complex toward the uropod of the cell and "cap formation" have been described by various investigators (McFarland and Schechter, 1969; Raff et al., 1970; Smith and Hollers, 1970; Taylor et al., 1971). Pinocytosis occurs within the first minutes after exposure to the antibody or lectin (Hirschhorn, 1969; Hirschhorn et al., 1970; McFarland and Schechter, 1969; Smith and Hollers, 1970; Taylor et al., 1971), followed by a discharge of lysosomal hydrolases (Hirschhorn et al., 1968; Hirschhorn et al., 1970; Hirschhorn and Hirschhorn, 1965; Nadler et al., 1969) and m-RNA synthesis (Baserga and Stein, 1971;



Hirschhorn, 1969). The synthesis of new proteins begins a few hours after the initial binding of the antigens or lectins to the cell membrane. The importance of lysosomal-induced changes in the cell periphery in the stimulation of mitosis of normal cells (Allison, 1969), or as a regulatory factor in cell growth has been proposed by many investigators (Dingle, 1969; Weiss, 1967). Further support for the concept of enzyme modification of the cell periphery comes from the recent finding that treatment of cultures of contact inhibited normal cells with proteases not only rendered them susceptible to agglutination by lectins but also stimulated bouts of cell division (Burger, 1971; Sefton and Rubin, 1970). However, the modification of the cell membrane by lysosomal enzymes is probably not required for adherence of cells induced by alloantibodies, since the presence of hydrocortisone which inhibits the release of lysosomal enzymes does not inhibit the adherence of cells. The mechanism of cell adherence induced by alloantibodies is therefore different from the cell stimulation which occurs concurrently with the cell adherence or agglutination with some lectins or antibodies.

Protein synthesis and DNA synthesis may not be required for the adherence of cells induced by alloantibodies. This is in accord with the report by Frye and Edidin (1970) that the reorganization of membrane was possible even in the presence of protein and nucleic acid inhibitors. This supports the proposed fluid mosaic model of cell membrane by Singer and Nicolson (1972), according to which phospholipids and proteins do not have a fixed location in the membrane but diffuse laterally in the membrane, without necessitating the synthesis of new membrane.





## Chapter 3

### Allofixation Properties of Chicken Alloantibodies

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## Introduction

It has long been known that chicken antibodies and complement are unusual. In mammals, different antigen-antibody systems are known to exhibit different abilities to fix guinea pig complement (Kabat and Meyer, 1948) and failure of immune chicken sera to show significant complement fixing activity was mentioned by Meyer (1944) and by Rice (1947) for chicken infected with Ornithosis virus and S. pullorum. The fact that high agglutination titered immune chicken sera are unable to lyse foreign red blood cells (RBC) in the presence of complement has been described by Janković and Isaković (1960). They were unable to lyse duck and rabbit RBC sensitized with immune chicken antibody in the presence of complement from many species of animals. Conversely, the naturally occurring and "immune" hemolysins of several mammalian species were unable to utilize chicken complement, provided that any naturally occurring hemolysin in the chicken serum were first removed (Rose and Orlans, 1962). When chicken RBCs were used as the indicator system, no lysis was obtained with naturally occurring hemolysins of six mammalian species in the presence of chicken complement. It was postulated that chicken antibody and antigen formed non-complement-fixing aggregates and chicken antibody probably lacked some character that was important for combining complement (Meyer and Eddie, 1956; Rice, 1948). There seems to be at least a partial incompatibility between mammalian antibodies and chicken complement.

Physico-chemical studies of chicken antibodies (Tenenhouse and Deutsch, 1966; Orlan et al., 1961) also revealed that chicken



immunoglobulins possessed a high electrophoretic mobility at pH 8.6, a higher sedimentation coefficient, a lower isoelectric point and a higher hexose content than most mammalian species. All these differences between the chicken antibody and its mammalian counterpart may be associated with incompatibility of the antibodies and complements of these two classes of animals. However, chickens produce different classes of antibodies in the primary and hyperimmune antisera. Physicochemical studies revealed that primary serum contained 7S nonhemagglutinating "univalent" (Orlans et al., 1961) and 19S hemagglutinating antibodies, and the hyperimmune sera contained mostly 7S hemmagglutinating antibodies. Mercaptans inactivated primary precipitins and only partially inactivated hyperimmune precipitins. Because of these great differences in antibody species in the primary and hyperimmune sera, I investigated the effects of primary, secondary and tertiary sera on the adherence or fixation of cells to surfaces with alloantibodies. Their relationship with the antigen-antibody system detected by hemagglutination was compared. The peculiarity of chicken complement and antibodies and their incompatibility with mammalian antibodies and complements also led me to investigate the interaction of chicken and mammalian antibodies with their complements.





## Materials and Methods

### I. Preparation and Treatment of Alloantisera

The antisera were prepared by conventional immunization as described earlier (see Chapter 1). Anti-B<sub>14</sub> sera were produced by injecting  $\underline{B}^{14}/\underline{B}^{14}$  whole blood into  $\underline{B}^1/\underline{B}^2$  or  $\underline{B}^2/\underline{B}^2$  recipients; and anti-B<sub>2</sub> sera were produced in  $\underline{B}^1/\underline{B}^{14}$  or  $\underline{B}^{13}/\underline{B}^{13}$  recipients. Three injections of 2 ml. whole blood were made on alternate days and the antisera obtained 5 to 7 days after the last injection were referred to as primary antisera. The secondary antisera were collected one month later after 2 injections of 2 ml. whole blood to the same recipients. Further immunizations were performed on a monthly basis on the same recipients for a period of time, if necessary, with the blood of the same donors. Antisera collected after a second set of immunizations were referred to as hyperimmune sera.

Some antisera were treated with 2-mercaptoethanol at room temperature for one hour. Equal amounts of antisera and 0.1M 2-mercaptoethanol (ME) were incubated in a test tube, and used directly without further dialysis for the allofixation test. The allofixation test was performed as described in the chapter dealing with the methodology of allofixation. HBSS supplemented with 15mM HEPES buffer and pH adjusted to 8.5 with 0.1N sodium hydroxide solution was used as the diluent for the test.

Partially purified immunoglobulins (Ig) were obtained by 3 separate salt precipitations in 18%, 14% and 14% sodium sulfate solution at room temperature. Equal amounts of antisera and sodium sulfate



solution were mixed together with rapid stirring. The mixture was allowed to stand at room temperature for 30 minutes before centrifugation at 3-5,000 rpm. The precipitates were collected and resuspended in borate-buffered saline (BBS, pH 8.2). The final preparation was either dialyzed or used directly for further purification in Sephadex G-200 column. Partially purified IgM and IgG were obtained by passing the salt precipitated Ig through the gel column, and the protein concentrations were measured at 280 m $\mu$  with a Beckman Spectrophotometer. The purity of the fractions was tested with goat anti-chicken-serum by the immunoelectrophoresis method.

## II. Preparation and Treatment of Heterologous Antisera and Plasma

Chicken anti-mouse RBC-serum was prepared by injecting 1 ml. whole mouse blood into the wing vein of chickens. The animals were bled one week later with heparin as anticoagulant. The serum was stored at -20°C until used. Goat anti-chicken RBC-sera were obtained from goats immunized with chicken RBCs and were stored at -20°C.

Fresh chicken plasma was absorbed with mouse RBCs at 4°C to remove the naturally occurring antibodies. This absorbed chicken plasma was stored at -20°C and used for the fixation test of mouse cells. Fresh mouse plasma was obtained by heart puncture. Heat inactivation of antisera and fresh plasma were done at 56°C for 30 minutes and the precipitates were removed by high speed centrifugation.

## III. Preparation of Cell Suspensions

Chicken lymphocytes were obtained from the peripheral blood of





chicken as described previously. Mouse lymphoid cells were obtained from the lymph nodes of mice. The mouse was killed by cervical dislocation or by ether and the lymph nodes from the axillary, branchial, inguinal and mesenteric regions were removed with fine forceps. The fatty tissues were removed and discarded. The organs were rinsed with HBSS and the clean lymph nodes were teased with fine forceps in a petri dish containing HBSS to release the lymphocytes. The cells were collected into tubes and washed with HBSS several times by centrifugation. The cells were then incubated with trace amounts of autologous fresh plasma at 37°C for 30 minutes to remove the adherent cells. Several such incubations may be required in some cases to remove all the adherent cells. The non-adherent cells were collected and washed with HBSS. The cell concentration was adjusted to  $4-6 \times 10^6/\text{ml}$ .

#### IV. Cell Adherence Test

The fixation of cells was performed as described in detail before (see Chapter 1). Fifty  $\mu\text{l}$  of cell suspension was added to a tube containing 25  $\mu\text{l}$  fresh plasma, 25  $\mu\text{l}$  antiserum and 100  $\mu\text{l}$  HBSS supplemented with HEPES (15mM) and the pH was adjusted to 8.5 with 0.1N NaOH. The incubation was done at room temperature for one or two hours. The number of cells was counted at "zero" time and was recounted after the incubation by hemocytometer or cell counter. The loss of cells was determined and the percent of adherent cells was calculated.

#### V. Hemagglutination Test

The hemagglutination test (HA) was performed with plastic



hemagglutinin titer plates (Microbiological Associates). Micro-pipettes (25 ul) were used to pipette antisera and salt solution (0.85% saline). Usually each of the wells was filled with 25 ul of saline and the first well was then filled with 25 ul of antiserum. Microdilutors (25 ul, Cook Engineering) were used to dilute the antiserum from one well to the other by simply transferring the dilutors from one well to the next. Twenty-five ul of 5% RBC in saline was added to each well with a micropipette and the mixture was shaken thoroughly. The results of agglutination were read after 30 minutes and repeated shaking was needed in some cases for detection of weak cross-reactions.



## Results

### (a) Allofixation and Hemagglutination with Chicken Alloantibodies

#### I. Allofixation and Hemagglutination with Primary, Secondary and Tertiary Antisera

Allofixation of lymphocytes is very dependent on the type of antisera employed in the test (Figure 41a, b). Primary antisera obtained after 3 injections of 2 ml. whole blood are usually weak in allofixation (AF) or do not react at all, though the hemagglutination (HA) titer may be quite high. Three types of primary antisera may be obtained from the first immunization of chicken with alloantigens. Quite frequently, antisera do not give any hemagglutination and AF reactions after primary immunization, even though a major histocompatibility difference was involved. These antisera are not used for AF test if the HA titer is less than 1/4. The poor production of antibody may be due to genetic differences in the response to antigens by individual animals. The second type and most frequently obtained primary antisera are weak in AF but are quite strong in the HA reaction (Figure 42a, b, c). This suggests that the species of antibody responsible for AF is different from that causing the HA reaction. The third type of primary antisera induces quite a strong AF in conjunction with the HA reaction (Figure 42d). This suggests the presence in these antisera of a new type of antibody which is probably different from that causing HA reaction. The strength of AF is always weaker than the HA reaction of the same primary antiserum, and the ratio of AF to HA titer is therefore less than 1.0 (Table 4). This appears to be true





for anti-B<sub>2</sub> sera produced in  $\underline{B}^1/\underline{B}^{14}$  or anti-B<sub>14</sub> sera produced in  $\underline{B}^2/\underline{B}^2$  and  $\underline{B}^1/\underline{B}^2$  recipients, but anti-B<sub>13</sub> produced in  $\underline{B}^1/\underline{B}^{14}$  or  $\underline{B}^2/\underline{B}^{14}$  recipients have AF/HA ratios equal or greater than 1.0.

The secondary antisera which were obtained from the same birds one month later with two more injections of 2 ml. whole blood are very different from the primary antisera. These antisera are strong in both AF and HA reactions, and the AF/HA ratios of anti-B<sub>2</sub> and anti-B<sub>14</sub> sera are approximately 1.0 (Table 4). This AF/HA ratio may be characteristic of anti-B<sub>2</sub> and anti-B<sub>14</sub> sera or it may be just a coincidence. The AF/HA ratio of anti-B<sub>13</sub> is found to be greater than 1.0, and one anti-B<sub>21</sub> produced in a  $\underline{B}^1/\underline{B}^1$  recipient also has an AF/HA ratio greater than 1.0 (Table 4). The strong AF reaction derived from the secondary antisera suggests a great increase in the presence of allofixing-antibody in the secondary antisera. The production of allofixing-antibody is further increased in the tertiary immunization, but the HA-reacting antibody remains almost constant after secondary immunization (Figure 42). The AF/HA ratios are greatly increased in the tertiary antisera due to the increase in strength of the AF reaction and without further increase in the HA reaction. The increase in the AF reaction of the tertiary antisera may be due to either increased amounts of allofixing-antibody or to increased avidity or affinity for antibody to the antigens. The AF/HA ratio for anti-B<sub>2</sub> and anti-B<sub>14</sub> sera of the tertiary antisera is always greater than 1.0 (Table 4).

The reactions of some primary antisera in the allofixation test may be due to the number of cross-reactions that the antisera may have. Table 4 demonstrates that the AF reaction is not correlated to the



number of factors that an antiserum reacts with. For example, one anti-B<sub>14</sub> (28464/1260) serum which was produced in a  $\underline{B}^1/\underline{B}^2$  bird shows cross reaction only weakly with some of the B<sub>15</sub> cells, yet the AF reaction is almost as strong as that of HA. On the contrary, anti-B<sub>14</sub> (28464/28482) serum produced in a  $\underline{B}^2/\underline{B}^2$  bird shows cross reactions with B<sub>1</sub>, B<sub>13</sub>, B<sub>15</sub> and B<sub>21</sub> in the HA tests but the AF reaction is not detected at a 1/4 dilution. This may suggest that primary antisera with more cross reactions may be weak in each case for a particular specificity and this affects the AF reaction. However, this is proved not to be true, since an anti-B<sub>14</sub> (1276/942) serum which shows cross reactions with B<sub>1</sub>, B<sub>13</sub> and B<sub>15</sub> and was produced in a  $\underline{B}^2/\underline{B}^2$  bird shows both AF and HA reactions. This result also indicates that the different combinations of immunization do not determine the type of antiserum produced, since anti-B<sub>14</sub> (28464/28482 and 1276/942) sera produced in different  $\underline{B}^2/\underline{B}^2$  birds may or may not produce allofixing-antibody in the primary antisera.

The secondary and tertiary antisera are completely different from the primary antisera in both their capability to fix cells and in the cross reactions detected by the AF and HA tests. The secondary and tertiary antisera show the same pattern of cross reactions in HA and AF tests (Table 5a, b). However, the strength or titer of cross reactions detected by HA and AF tests are different and the cross reactions increase in strength with increasing number of immunizations. For the anti-B<sub>2</sub> produced in  $\underline{B}^1/\underline{B}^{14}$  bird, the cross reaction for AF is very strong for B<sub>13</sub>, intermediate for B<sub>21</sub> and weak for B<sub>15</sub> antigens in both secondary and tertiary antisera. On the contrary, the strength of cross reactions for B<sub>15</sub>, B<sub>13</sub>, and B<sub>21</sub> are almost the same in HA tests





though  $B_{15}$  is slightly stronger than  $B_{21}$  and  $B_{13}$ . The  $B_{13}$  is not only weak in cross reaction detected by the HA test but the anti- $B_{13}$  specific antisera are also weak in the HA test, though their allofixation titers are very strong (Table 4). This indicates  $B_{13}$  alloantigen probably favors the production of allofixing-antibody or the antigen is present in great amounts on the lymphocyte membrane. The anti- $B_{21}$  produced in a  $\underline{B}_1^1/\underline{B}_1^1$  bird shows strong cross reactions with  $B_2$ ,  $B_{13}$ ,  $B_{14}$ , and  $B_{15}$  cells for both AF and HA reactions (Table 5b) and the removal of these cross reactivities leaves only a weak HA and AF activity for  $B_{21}$  cells.

Table 6 illustrates that anti- $B_{14}$  produced in a  $\underline{B}_2^2/\underline{B}_2^2$  bird cross reacts with  $B_1$  and  $B_{13}$  antigens but does not react with  $B_2$  antigens. The absorption of this antiserum with  $B_1$  and  $B_{13}$  erythrocytes produced antiserum that is specific to  $B_{14}$  with the HA test, but the AF reactions for  $B_1$  and  $B_{13}$  are still present (Table 7). The  $B_1$  cross reaction is stronger than the  $B_{13}$  in this absorbed serum. This shows that the specificity of antiserum as shown by the HA test may not necessarily indicate the specificity for AF reactions. The HA reaction may have only limited use in defining the specificity of the AF test. The difference in the detection of alloantigens by the HA and AF tests is further demonstrated by the anti- $B_{14}$  serum produced in a  $\underline{B}_{13}^{13}/\underline{B}_{13}^{13}$  bird, and absorbed with  $B_1$  and  $B_2$  erythrocytes (Table 8). The absorption is complete when the HA test is run immediately but the cross reactions for  $B_1$  and  $B_2$  reappear later. When this antiserum was tested for cross reaction with  $B_1$  and  $B_2$  cells with AF test, the results were negative. This suggests that the allofixing-antibody has been completely absorbed but the HA-reacting antibody has not. This tends to strengthen my



speculation that HA and AF reacting antibodies are two different species of antibodies.

The allofixing-antibody is probably associated with the 7S immunoglobulins of the antiserum. Figure 43 shows that anti-B<sub>14</sub> serum treated with an equal volume of rabbit anti-chicken IgG-serum at 37°C for 30 minutes completely loses allofixing-antibody. This suggests that IgG or 7S antibodies are effective in the AF-reaction.

## II. Effect of Mercaptoethanol on Allofixation and Hemagglutination of Primary, Secondary and Tertiary Antisera

I have noted in the previous experiments that primary, secondary and tertiary antisera differ in their ability to induce AF and HA reactions. There are at least two species of antibodies, one responsible for the strong AF and the other for the primary HA reactions. After treatment with 0.1M mercaptoethanol (ME) at room temperature for one hour, I am able to differentiate between the allofixing-antibodies present in the primary and hyperimmune sera. Figure 44 shows that primary antisera contain allofixing-antibody sensitive to ME. The fixation of the cells to surfaces is reduced to approximately none after the ME treatment. However, the hyperimmune antisera are very resistant to the treatment by ME. The fixation of cells in the treated antisera is almost as effective as in the untreated antisera at high concentration of antibodies, though a slight decrease in AF reaction is noted at high dilution of antibodies. The effect of ME on AF reaction is very consistent for all the primary antisera tested. Table 9 illustrates that the AF reactions of all the 4 primary antisera tested are completely inhibited by ME, though the HA titer of the





antisera is only slightly reduced. The AF and HA of all the 8 hyperimmune sera tested were resistant to the ME treatment. The  $HA_{me}/HA_c$  ratio of most antisera is 0.75. However, some antisera which have low HA at the beginning are reduced to even lower HA after ME treatment, and the  $HA_{me}/HA_c$  ratio becomes quite low, i.e., 0.25 or lower. The effect of ME on primary antisera is shown in the  $AF_{me}/AF_c$  ratio (i.e., AF titer of ME treated antiserum versus AF titer of untreated antiserum). The values of all the primary antisera tested are approximately equal to zero. The actual value is not possible to determine because the lowest dilutions tested are 1/4 or 1/8 of the antisera. On the contrary, the  $AF_{me}/AF_c$  ratio is between 0.75 and 1.00 for the hyperimmune sera. This indicates that most of the allofixing-antibodies in the hyperimmune sera are very resistant to ME and the slight decrease of AF activity in these antisera may be due to the inactivation of the same species of allofixing-antibody found in the primary antisera. Thus the data appear to favour the presence of two species of antibodies for AF reaction. One is present in the primary antisera and is very sensitive to ME, the other species is present predominantly in the hyperimmune sera and is resistant to the reducing agents. The HA-reacting antibody is probably different from the two species of allofixing-antibodies.

The sensitivity of allofixing-antibody found in the primary antisera may be due to either the low amounts of such antibody present in the primary antisera or the inhibition of the antibody by the high concentration of ME in the incubating medium. Figure 45 illustrates that addition of 5mM of ME to the test medium almost completely





inhibits the AF reaction of the primary antisera though the AF reaction of hyperimmune sera is not affected. This difference in sensitivity to ME by primary and hyperimmune sera may be due to low amounts of allofixing-antibody present in the primary antisera. However, this does not exclude the possibility that the allofixing-antibody found in primary antisera is a different species of antibody, since the same concentration of ME does not inhibit the AF reaction induced by hyperimmune sera. If the effect of ME on AF reaction comes from the high concentration of ME in the test medium, the same inhibition will be seen with the hyperimmune antisera. This appears to be unlikely and thus the production of two species of allofixing-antibodies by chicken still seems likely.

### III. Allofixation and Hemagglutination with Salt-Precipitated Immunoglobulins and Partially Purified IgM and IgG Antibodies

Salt-precipitated globulins from hyperimmune sera, after dialysis for 48 hours at 4°C with 3 changes of borate-buffered saline (BBS, pH 8.2) were tested for the adherence of cells. Table 10 shows that salt-precipitated globulins contain both antibodies and synergistic factors. At 1/4 dilution of globulins, fixation of cells occurs in HBSS, BBS and fresh chicken plasma. The fixation of cells does not occur in the presence of heat inactivated chicken plasma. The fixation of cells is complete in fresh chicken plasma, and only partial fixation occurs in HBSS and BBS. This suggests that the synergistic factors are limited in the tests with HBSS or BBS and salt-precipitated globulins. Supplement of these factors with normal fresh plasma corrects this. The inhibition by heat inactivated plasma may be due to an anticomplementary



effect. Allofixation may be complete for the untreated antiserum whether the diluent is HBSS, BBS or fresh chicken plasma. A partial fixation also occurs with heat inactivated plasma. This indicates that the synergistic factors are in excess in the untreated antiserum, and the antagonistic effect of heat inactivated plasma is not strong enough to counter all the synergistic activity.

The heat-inactivated, salt-precipitated globulins do not fix cells with HBSS or BBS, but the fixation is complete with the addition of fresh chicken plasma. This indicates that heat inactivation is a very efficient way of removing synergistic factors.

Partially purified IgM and IgG fractions were obtained from the passage of salt-precipitated globulins through a Sephadex G-200 column. The fixation of cells by these fractions of antibodies was performed with the supplement of fresh chicken plasma at pH 8.5. Table 11 illustrates that both the IgM and IgG fractions are able to induce cell adherence. The HA titers of IgM and IgG are  $1/8 \pm 16$  and  $1/32 \pm 64$  respectively, but the AF titers are  $1/32$  and  $1/512$ . This indicates that most of the antibody activity is located in the IgG fraction; the reaction found in the IgM fraction may be due to contaminating IgG. Highly purified IgG and IgM are therefore needed to confirm this speculation. Immuno-electrophoresis tests of these globulin fractions with goat anti-chicken-serum were inconclusive.

(b) Interaction of Chicken and Mammalian Antibodies and Complement in Fixation of Cells

Goat anti-chicken RBC-serum was tested with peripheral lymphocytes of chicken. The result is shown in Figure 46. Goat anti-chicken





red cell-serum diluted with HBSS does not induce fixation or adherence of cells, and no cytotoxicity of cells is seen. However, in the presence of fresh chicken plasma, both fixation and cytotoxicity are found. At high concentrations of antiserum, complete lysis of cells is seen and fixation of cells is seen only at high dilutions of antiserum. At intermediate concentrations of antiserum, the fixation of cells is associated with cytotoxicity, though not all the dead cells adhere. These data indicate that the lysis of cells at high concentrations of goat antiserum, with fresh chicken plasma, is probably due to the combined effect of components from both species. Presumably it is dilution of goat complement at high dilutions of antiserum which accounts for the loss of cytotoxicity. The fixation seen under these conditions may represent the combination of goat antibody and synergistic component of chicken serum.

When the antiserum was heated at 56°C for 30 minutes to inactivate the goat complement, agglutination was observed with the test in HBSS. The addition of fresh chicken plasma to this heat inactivated goat antiserum induces both fixation and cytotoxicity as in the untreated antiserum, except that the toxicity is reduced. This suggests that heat reduces but does not completely remove certain goat complement components in the antiserum. The occurrence of some fixation and cytotoxicity of cells at high concentration of heat inactivated goat antiserum in HBSS is seen. This is probably due to the trace amounts of fresh chicken plasma which were added to the cell suspension to prevent aspecific adherence.

Chicken anti-mouse red cell-serum was tested with mouse lymph



node cells. Table 12 illustrates that chicken antibody directed against mouse red cells is able to induce fixation of mouse lymph node cells. The fixation of the lymph node cells is accompanied by cytotoxicity. Heat inactivated chicken anti-mouse red cell-serum is not toxic to the mouse cells, but agglutination interferes with fixation of cells when tested without the supplement of fresh chicken plasma. However, the supplement of fresh chicken plasma to heat inactivated chicken anti-serum does not enhance the fixation of cells to their original level. The fixation of cells is weak and is associated with either agglutination or cytotoxicity. This suggests that the heat sensitive components found in the immune serum may inhibit the normal fresh serum.

The anticomplementary effect of mammalian fresh plasma on fresh chicken plasma was detected with the presence of autologous mouse plasma in fixation of mouse lymph node cells by chicken anti-mouse red cell-serum. Figure 47 illustrates that the fixation of cells is partially or completely inhibited depending on the amount of mouse serum present in the test medium. The higher the amount of serum, the lower is the fixation. In 25% mouse serum, almost complete inhibition is seen at 1/16 dilution of the antiserum, and only about 50% of cells adhere at 1/8 dilution. About 90% of cells adhere at 1/8 and 1/16 dilutions and 50% adhere at 1/128 dilution of antiserum without the addition of mouse serum. In 1% mouse serum, however, 90% of cells adhere at 1/8 dilution and 50% of cells adhere at 1/32 dilution of the antiserum. This result suggests that the mouse serum protects the cells from damage by heterologous antibody and complement, but the mechanism of the protection is not known.





## Discussion

### (a) Allofixation and Hemagglutination with Chicken Alloantibodies

The hemagglutination (HA) test does not always correlate with other antigen-antibody reactions. Dreesman et al. (1965) demonstrated that in chicken antisera produced by a single injection of bovine serum albumin (BSA) agglutinated tanned red blood cells coated with BSA. The macroglobulins were responsible for the HA reaction, and the IgG immunoglobulins were responsible for precipitin reactions. Secondary or hyperimmune IgG antibodies accounted for both reactions. These findings are in accord with our results on the effects of primary, secondary, and tertiary immunization on AF. Primary antisera do not usually cause AF reactions though HA reaction is detected. Secondary and tertiary antisera induce AF and HA reactions though the AF titers may exceed the HA titers.

The occurrence of hemagglutinins in most of the primary antisera is not correlated with AF. This suggests that HA and AF reactions are two different antigen-antibody reactions even though the same type of antigens may be involved. The high HA and weak AF reactions in most of the primary antisera are probably due to the production of low amounts of allofixing-antibody. The macroglobulin is more likely to cause HA reaction (Benedict et al., 1963b) and it probably represents all the HA reaction in the primary antisera, since the low molecular weight antibody present in the primary antisera does not cause HA reaction (Benedict et al., 1963a; Dreesman et al., 1965; Orlans et al., 1961). The low molecular weight antibody present in the





primary antisera may differ from the predominant antibody found in the secondary and hyperimmune sera. Primary 7S may be univalent (Orlans et al., 1961), non-hemagglutinating and non-precipitating in low salt, whereas 7S antibody found in the hyperimmune sera agglutinates sensitized red cells and precipitates in low salt (Benedict et al., 1963a, 1963b; Kubo and Benedict, 1969). It appears that only the 7S antibodies are able to induce AF reaction, but the efficiency of the 7S antibody present in the primary antisera is smaller than that in the secondary antisera. However, the mercaptoethanol treated experiments indicate that allofixing-antibody present in the primary antisera may be the 19S antibody due to its sensitivity to this reducing agent. The hyperimmune sera are only slightly affected by the ME treatment indicating that 7S antibody is involved instead of the 19S antibody. These results favor the presence of two antibodies able to fix cells. However, the actual proof has to come from the fractionations of these two species of antibodies.

The strength of AF is increased in tertiary antisera over the secondary antisera, but the HA titer remains almost constant. This indicates that either the amount of allofixing-antibody is increased in tertiary antisera or the avidity or affinity for the antigens is increased. The maintenance of a constant level of the HA reaction is in accord with the report that macroglobulin antibodies in chicken are constantly produced after primary immunization and the amount of macroglobulin antibodies produced does not diminish after primary immunization in the chicken in contrast to the mammalian systems where the synthesis of macroglobulin antibodies does diminish (Bauer and Stavitsky, 1961; Benedict et al., 1962; Stelos, 1958).

The HA and AF cross reactions for B antigens are similar when



secondary and tertiary antisera are used. This suggests that the HA reaction may be used as a marker for AF reaction, and suggests a similarity of antibodies involved in AF and HA reactions. However, this appears to be unlikely, since HA is caused mainly by the 19S antibodies and only partially by the 7S antibodies (Benedict et al., 1963b). My results show a difference in the strength of the cross reactions for different B antigens in HA and AF tests. The strength of cross reactions for HA does not correlate with that detected by AF and the difference is so great that it is unreasonable to assume that the reactions are due to the same antibodies. However, the discrepancy between the reaction pattern of AF and HA reactions may be due to the difference in the distribution of different B alloantigens on the surfaces of erythrocytes and lymphocytes. The difference in the distribution of alloantigens on the cell membrane of erythrocytes and lymphocytes is well known. In chicken, antigens of the B and C systems have been demonstrated by lymphoagglutination and absorption of anti-erythrocyte sera with lymphocytes, but antigens of the D and L systems have not been found (Schierman and Nordskog, 1962). In addition to these 2 systems, there are antigens which do not seem to be detectable on red blood cells (McDermid, 1968; Thein and Schmid, 1968).

The ability of salt-precipitated globulins to induce AF reactions and the location of major antibody activity in the partially purified IgG fraction are in favour of the 7S antibody as the allofixing-antibody found in the hyperimmune sera. The complete inhibition of AF reaction by rabbit anti-chicken IgG-serum further supports IgG as the antibody responsible for AF. The heat inactivation of fresh plasma





in association with the loss of AF activity also favours the participation of chicken complement in allofixation.

(b) Interaction of Chicken and Mammalian Antibodies and Complement in Fixation of Cells

If complement plays a role in the fixation of mammalian cells, fixation and lysis should parallel each other to some degree. The chicken's natural antibodies for rabbit erythrocytes lyse these cells when supplemented with avian or with some fresh mammalian sera. Conversely, the chicken's natural antibodies for duck erythrocytes do not lyse these cells when supplemented with avian sera, but will do so when supplemented with some fresh mammalian sera (Janković and Isaković, 1960). This contrast may be presumed to reflect relatively high levels of natural antibodies to rabbit cells and relatively high levels of complement in some mammalian sera. When chickens were immunized to increase the levels of antibodies to rabbit and duck cells there was an increase in hemagglutinating antibody, but no increase in hemolytic antibody with either fresh chicken or mammalian sera. Presumably, this failure is associated with the fact that the induced hemagglutinins are likely to be 7S immunoglobulins which may be less effective hemolysins than the natural antibodies, presumed to be 19S immunoglobulins. Rose and Orlans (1962) failed to restore hemolytic activity for sheep erythrocytes to heated immune chicken sera by addition of guinea pig complement, although they succeeded in doing so by adding chicken complement. These authors substantiate Janković and Isaković's report that mammalian sera enhance the hemolysis of mammalian cells by natural chicken antibodies and chicken complement. However, they attribute



this to mammalian antibody instead of mammalian complement (Rose and Orlans, 1962).

My observations indicate that fresh chicken plasma does not restore lytic and "fixation" activity to heated, immune chicken serum when the target cell is the mouse lymphocyte. This failure is concordant with Janković and Isaković's failure to restore hemolytic activity (for duck erythrocytes) to heated immune chicken sera, and would appear to differ from Rose and Orlans' report of successful restoration of hemolytic activity by addition of fresh chicken serum. Direct comparison is vitiated, however, by the differences in the tests; one group used duck erythrocytes and the other used sheep erythrocytes as the target for their crucial experiments. In tests of avian complement with avian cells comparison is complicated by the selection of species; one group used fresh turkey serum as a source of complement and the other group used seven avian species, but not the turkey. The failure to restore lytic and fixing activity to chicken antibodies with avian complement when mouse lymphocytes are the target lacks, therefore, a fully appropriate hemolysis model.

Cross-class differences in complement have also been described for hemolysis reactions based on natural mammalian antibodies, avian complement and avian erythrocytes. Fresh chicken serum was an ineffectual source of complement for the hemolysis of chicken cells by natural mammalian antibodies (Rose and Orlans, 1962). In my experiment, however, fresh chicken plasma restores lympholytic activity to a strong, heated immune goat antiserum. When the goat antiserum is tested in a succession of dilutions, lytic activity decreases and disappears and fixing activity





increases and, at high dilution is the only remaining activity. Thus in this mammalian anti-avian reaction avian complement seems to play a role which cannot be attributed to the presence in fresh plasma of natural antibodies to another class of cells.

It has been observed that heated antisera of certain domestic chickens does not fix guinea pig complement in the direct complement fixation test although unheated sera may (Rice, 1947). Chicken C'1 in the unheated serum is probably required to activate guinea pig complement before the fixation of guinea pig complement by chicken antigen-antibody complex (Bensen et al., 1961). The inability of heated chicken antiserum to utilize the complement from fresh chicken serum for the fixation or lysis of mouse lymphoid cells may be due to loss of some important components. The anticomplementary effect of heated serum on the fresh serum has been described by Hawkins (1961). He reported that heated guinea pig complement inhibited the lytic activity of fresh guinea pig complement. The anticomplementary effect or incompatibility between mammalian and fowl complements or between different mammalian species has been reported (Amiraian et al., 1962; Laporte, 1952; Orlans et al. 1962b; Rice and Crowson, 1950). Mixtures of chicken and guinea pig complements are less hemolytic than either complement by itself regardless of the nature of the indicator system (Orlans et al., 1962a). The inhibition or reduction of fixation and lysis of mouse lymphoid cells by chicken anti-mouse red cell-serum in the presence of fresh mouse serum may be due to the anticomplementary effect of the mouse serum on the chicken complement.





Chapter 4

Concanavalin A-Induced Cell Adherence

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## Introduction

The plant lectins, Concanavalin A (Con A) from Jack beans (Canavalia ensiformis) (Summer and Howell, 1936), Phytohemagglutinin (PHA) from Phaseolus vulgaris (Nowell, 1960) and Pokeweed mitogen (PWM) from Phytolacca americana (Farnes et al., 1964) induce the transformation of lymphocytes in culture (Perlmann et al., 1970; Robbins, 1964), but only Con A and PHA agglutinate erythrocytes (Summer and Howell, 1935; Takahashi et al., 1967). PHA agglutinates both normal and abnormal cells transformed by oncogenic virus or other agents, while Con A agglutinates only the transformed cells (Aub et al., 1965; Burger and Goldberg, 1967; Inbar and Sachs, 1969; Steck and Wallach, 1965). These properties of plant lectins are the result of the interactions of these phytomitogens with the specific, but apparently different, surface receptors on the cell membrane. The carbohydrate moieties of the cell membrane appear to be the receptor sites for these plant lectins (Goldstein et al., 1965; Leon, 1967). Con A binds specifically to carbohydrates containing terminal  $\alpha$ -methyl-D-mannoside,  $\alpha$ -methyl-D-glucoside and  $\beta$ -D-fructose residues of polysaccharide chains (Goldstein et al., 1969), while PHA binds specifically to N-acetyl-galactosamine residues of carbohydrates (Borgery et al., 1966).

The specific interaction of these plant lectins with the receptor sites on the cell membrane appears to be similar to the specific binding of the alloantibodies with the alloantigens on the cell membrane. This study deals with the induction of cell adherence to





surfaces by plant lectins or phytomitogens. PHA induces cell adherence and agglutination, Con A induces only cell adherence and PWM induces neither. The adherence of cells induced by plant lectins strengthens the impression that the interaction of any molecules, antibodies, phytomitogens, drugs, hormones or other, with the cell membrane may be manifested as "cell adherence." The adherence of bacterial cells, lymphocytes and other cells to human or primate red cells in the presence of antibody and complement is well known (Nelson, 1963). The adherence of human red cells to mammalian tissue cultures sensitized by specific antisera and complement has been described by Kano and Milgrom (1965).



## Materials and Methods

Lymphocyte suspensions were prepared from different lines of White Leghorn chickens as described previously (see Chapter 1). Thymus and bursa cells were obtained by teasing the thymus and bursa from 3-9 week-old chickens with fine forceps and passing the tissues through a stainless steel mesh. The cells thus collected were washed several times with HBSS and incubated at 37°C for at least 30 minutes to remove adherent cells. Several incubations were needed in some cases to remove all the unwanted cells. The non-adherent cells were collected and washed several times with HBSS in the presence of trace amounts of autologous fresh plasma to inhibit aspecific adherence. The cell concentration was adjusted to  $4-6 \times 10^6$  cells/ml.

The adherence test was performed as follows: 100 ul of HBSS containing 2 times the final concentration of plant lectins and 50 ul of fresh chicken plasma or agamma chicken serum were pipetted into each plastic tube with a 25 ul micropipette. Fifty ul of cell suspension was then introduced into each tube and the cell concentration of the mixture was counted by an automatic cell counter (Fisher Autocytometer II). The tubes were incubated at room temperature (25°C) for one and/or two hours, and the loss of cells was calculated from a recount.

The pH of the solution was adjusted with 0.1N NaOH or HCl as required. For testing the effects of inhibitors (sodium cyanide, iodoacetamide, 2-deoxy-D-glucose, chloramphenicol, cycloheximide), EDTA, hydrocortisone, specific inhibitors of Con A ( $\alpha$ -methyl-D-glucoside,



$\alpha$ -methyl-D-mannoside) and N-acetyl-glucosamine, 50  $\mu$ l of 4 times the final concentration of inhibitors or reagents in HBSS, 50  $\mu$ l of 4 times the final concentration of lectins, 50  $\mu$ l of fresh chicken plasma or agamma chicken serum and 50  $\mu$ l of cell suspension were used. HBSS (pH 7.2) was used throughout the experiments as the diluent of serum, plasma, inhibitors and antiserum. Cells were treated with Con A on a petri dish and the adherent and non-adherent cells used for either the "pock" test on the CAM of chick embryos or for the allofixation test.





## Results

### I. Cell Adherence with Plant Lectins

The results have shown that the plant lectins Con A and PHA, but not PWM, cause fixation of lymphocytes to surfaces in the presence of serum proteins (Figure 48a, b, c). Con A induces cell adherence without causing any agglutination, whereas PHA induces adherence and agglutination of lymphocytes in a small plastic tube. Agglutination may not occur in some tests with PHA; the result is inconsistent if small plastic tubes are used. Performance of the test with PHA in a petri dish completely eliminates the agglutination seen in the small test tubes. This suggests that cell-to-cell agglutination may be prevented by an increase in the surface area to which the cells can adhere. On the other hand, the tendency of one type of cells to adhere or agglutinate may finally depend on the surface properties of the cells and their substrates.

Figure 49a and b illustrate Con A- and PHA-induced cell adherence to the surface of a plastic dish. The adherent cells are considered to be viable and active because the cells are still refractile under the phase contrast microscope and continual projections of pseudopods are noted. The adherent cells are considered functionally intact because the cells adherent to cover slips form "pocks" on the CAM of the chick embryos. PWM does not induce cell adherence at the concentrations of the mitogen tested (Figure 48c). PWM is known to induce transformation of lymphocytes but does not possess the agglutinating activity



of PHA and Con A (Farnes et al., 1964). These results suggest that agglutinating components are required for the induction of cell adherence to surfaces. The agglutinating and mitogenic components of the lectins may represent two separate proteins rather than one protein with two different activities.

## II. Con A-Induced Cell Adherence

The effect of pH on the adherence of lymphocytes with Con A was tested from pH 6 to 10. Con A was used for the test because it was consistent in inducing cell adherence. Figure 50 shows that the adherence of lymphocytes induced by Con A in the presence of serum proteins is not affected by a change of pH; however, the length of incubation does affect cell adherence. More cells adhere after two hours of incubation than after one hour, whether the incubating medium is 25% agamma chicken serum or fresh chicken plasma. The number of cells adhering is greater in 25% agamma chicken serum than in the fresh chicken plasma at one and two hours of incubation. This indicates a stronger inhibition by fresh chicken plasma than by agamma chicken serum.

The effect of Con A concentration in 25% agamma chicken serum and fresh chicken plasma is illustrated in Figure 51. The results demonstrate that 100 ug/ml of Con A is the minimal amount required to induce maximal adherence of lymphocytes at two hours in 25% fresh chicken plasma or agamma chicken serum; however, the minimal concentration of Con A necessary to produce cell adherence is lower in agamma chicken serum than in fresh chicken plasma. Fifty ug/ml of Con A induces cell adherence in 25% agamma chicken serum but not in 25% fresh chicken





plasma. This indicates that the repulsion forces exerted between the cells and the surfaces are greater in fresh chicken plasma than in agamma chicken serum of the same concentration. These repulsion forces may be due to charges present on the proteins in the two solutions. The effect of Con A concentration and serum protein concentration is interdependent: an increase in serum protein concentration requires a corresponding increase in Con A and vice versa. The effect of serum protein concentration on the adherence of cells in the presence of a constant amount of Con A is illustrated in Figure 52. The results show that 25 ug/ml and 50 ug/ml of Con A are not sufficient to overcome the repulsion forces of 25% agamma serum, whereas in serum dilutions of 1 to 16 or higher, the cells are able to adhere. At 100 ug/ml, Con A induces some cell adherence in 25% serum, but less than that obtained with Con A concentrations of 100, 50 or 25 ug/ml in serum dilutions of 1 to 16 or higher. This suggests that serum proteins at various concentrations exert repulsion forces of varying strengths between the cells and surfaces. The higher the concentration of serum, the greater the repulsion forces exerted between the cells and the surfaces. The interaction of the cells with their respective agents probably produces some forces to counterbalance the repulsion forces before any adherence of cells can take place. These repulsion forces are probably electrostatic forces exerted by the protein molecules on either the cells or the surfaces of the substrates. It is known that protein-coated surfaces or the addition of proteins into a test medium tends to inhibit or reduce the tendency of cells to adhere or aggregate (Easty et al., 1960; Taylor, 1961).



The amount of Con A available for the binding of surface receptors may have an effect on the rate of adherence. Figure 53 shows that more cells adhere in the presence of Con A at 200  $\mu\text{g/ml}$  than at 100  $\mu\text{g/ml}$  at one and two hours under the same conditions. This suggests that the availability of Con A affects the rate of cell adherence, and the higher the number of Con A molecules present in the solution the greater the chance that they will react with their respective receptor sites on the cell surfaces within a specific period of time. Figure 53 illustrates the effect of cell concentration on cell adherence. One million cells per ml appears to adhere as effectively as  $7 \times 10^6$  cells/ml. The higher cell concentrations may be slightly more effective than the lower cell concentrations. This suggests that the amount of Con A molecules are in excess in these tests.

The rate of adherence of lymphocytes induced by Con A is influenced by the time of incubation and the temperature of the incubating medium. Figure 54 illustrates the rate of adherence at 50  $\mu\text{g/ml}$  of Con A in 1% fresh chicken plasma. The results show rapid adherence of cells in the first hour of incubation and complete adherence of cells by two hours at room temperature. Cell adherence can be detected as early as 15 minutes and increases rapidly thereafter. The rapid adherence of cells indicates that the interaction of the cell receptors and their specific agents is of a chemical nature, and the reaction is probably too rapid for the formation or synthesis of new cell products. The rate of adherence is greatly affected by a change of temperature. Figure 55 shows that cell adherence increases rapidly from 4°C to 25°C, while further increase in temperature only slightly increases the rate





of adherence. Cell adherence is blocked at low temperature and at 4°C no cells adhere to the surfaces. The optimal temperature for cell adherence lies between 25° and 37°C, where maximal cell adherence is seen. This is in accord with the findings that malignant cells are agglutinated by Con A at 24°C and not at 4°C (Inbar et al., 1971). The adherence of cells to surfaces is probably associated with a temperature dependent process, since the interaction of Con A and the cell membrane occurs at low temperature (Sällström and Alm, 1972).

Con A is known to bind specifically with the polysaccharides on the cell surfaces that contain free glucopyranoside or mannopyranoside. Cell adherence to surfaces in the presence of Con A will be inhibited by these sugars if the reaction is specific. Figure 56 shows that 0.05 M  $\alpha$ -methyl-D-glucopyranoside and  $\alpha$ -methyl-D-mannopyranoside inhibit cell adherence, whereas N-acetyl-glucosamine does not. This indicates that the reaction is specific and the cell surface receptor for Con A is a polysaccharide containing the above sugars.

The metabolic inhibitors iodoacetamide and sodium cyanide are inhibitory to some forms of immunocytoadherence (Nelson, 1963; Siegel, 1972). The inhibitory effects may be due to the inhibition of key enzymes or to unknown interactions. Figure 57 illustrates the inhibitory effect of both iodoacetamide and sodium cyanide. The inhibition is detected with the addition of 1 mM of sodium cyanide and iodoacetamide; iodoacetamide appears to be a more effective inhibitor than sodium cyanide at this low concentration. The inhibitory effect is complete at 3 mM iodoacetamide but not with 3 mM sodium cyanide. Iodoacetamide is an alkylating agent and an inhibitor of the glycolytic





pathway, while sodium cyanide inhibits oxidative phosphorylation. The complete inhibition obtained with iodoacetamide may be due to a total blocking of the energy supply required for cell adherence, and the incomplete inhibition by sodium cyanide may be due to alternative energy supply routes which bypass the pathway blocked by sodium cyanide. However, inhibition by iodoacetamide through blockage of the glycolytic pathway cannot be confirmed by other inhibitors. An inhibitor of glucose metabolism, 2-deoxy-D-glucose, has no effect on Con A-induced cell adherence (Figure 58). This suggests that an interference with glucose metabolism may not be the actual cause of inhibition by iodoacetamide which reacts with several kinds of chemical groups, but is best known for its alkylation of sulfhydryls or mercaptans. Alkylation of certain surface receptor sites for Con A may alter the interaction of Con A with the surface receptors.

The inhibitors of protein synthesis, chloramphenicol and cycloheximide, were tested at their physiological concentrations. Twenty-five to 100 ug/ml of chloramphenicol (Figure 59) and 0.5 to 10 ug/ml of cycloheximide (Figure 60) do not inhibit cell adherence. This suggests that the synthesis of new protein is not required for cell adherence of this type. Some types of cell aggregation and adhesion are known to require the appearance of new substances outside the cell membrane before adhesion and spreading can occur (Rosenberg, 1960; Rosenberg, 1962; Takeichi, 1971; Takeichi and Okada, 1972; Taylor, 1961). The lymphocytes whose adherence is induced by Con A do not spread.

Cell membranes can be stabilized by treatment with hydrocortisone (Dingle et al., 1967; Fell et al., 1966; Fell and Weiss, 1965).



It blocks the release of lysozyme from lysosomes within the cells and lysozyme is thought to be associated with the modification of cell membrane which may be important for cell adherence. The effect of hydrocortisone on Con A-induced cell adherence was tested with 50 to 200 ug/ml of hydrocortisone-21-sodium succinate. Figure 61 shows that 50 ug/ml of hydrocortisone has no effect on cell adherence, but a slight inhibition is noted at 100 and 200 ug/ml. Since the occurrence of inhibition is associated with the toxicity of the drug on the cells, the effect of high concentrations of hydrocortisone may be misleading. An association between inhibitory and toxic effects of EDTA on Con A-induced adherence has also been observed (Figure 57). Three mM EDTA inhibits cell adherence and is also toxic to the cells. Death of the cells is accompanied by loss of activity and change of refractility. This confirms the speculation that adherence requires living and intact cells with active membranes. Energy is probably required for such activity, which may be temperature-dependent.

Con A-induced cell adherence is of no practical value unless it provides some genetic information regarding the type of cells tested. To investigate this problem, peripheral lymphocytes from different lines of chicken, the  $\underline{B}^2$ ,  $\underline{B}^{13}$ ,  $\underline{B}^{14}$ ,  $\underline{B}^{15}$ , and  $\underline{B}^{21}$ , were tested against 200 ug/ml, 100 ug/ml and 50 ug/ml of Con A in the presence of 25% serum. Table 13 indicates that the percentage of cell adherence is not affected by the lines of chicken use. It may be that the present experimental conditions used are not able to detect the difference or that the number of Con A receptor sites present on the lymphocytes of these genetic lines are the same.





The number of receptor sites for Con A may differ among cell types derived from different lymphoid organs. If we are able to detect these differences, this might facilitate the separation of cells of thymus and bursa origin in the peripheral blood. This possibility was tested with chicken bursa and thymus cell suspensions against different concentrations of Con A. Table 14 shows the results of 3 tests conducted with 3 different chickens. The results indicate that in the presence of 25% agamma chicken serum, bursa cells require a lower concentration of Con A than do thymus cells or peripheral lymphocytes to adhere to the surface. One hundred ug/ml or more are necessary for the thymus or peripheral lymphocytes to adhere under the conditions tested, whereas only about 25 ug/ml of Con A is required by the bursa cells. These results suggest that the bursa cells may contain a greater number of receptor sites on their surfaces than do the thymus or peripheral lymphocytes. Autoradiography may enable us to confirm this.

The binding of Con A to the cell surface may hinder the reaction of other receptors or antigens on the cell surface with their respective agents, if they are located on the same or neighbouring molecules or the interaction may cause steric interference with other antigen-antibody bindings. Con A and PHA have been shown to interfere with the binding or expression of cytotoxic activity of murine alloantisera (Ray and Simmons, 1973). This was investigated by treating lymphocytes with Con A in a petri dish at room temperature or at 4°C for one hour. The adherent cells were released by vigorous agitation with a pasteur pipette. The washed adherent cells and non-adherent cells obtained by



incubation at room temperature and at 4°C respectively were used for the allofixation test. Figure 62 shows that Con A-treated cells adhere as effectively as untreated cells in the presence of the same alloantibodies, and no significant difference between the treated and untreated cells has been found. However, in the absence of alloantibody, Con A-treated and washed cells do not re-adhere in the presence of either agamma chicken serum or autologous fresh plasma. The results indicate that binding of Con A to the cell surface does not interfere with the binding of alloantibodies to antigens on the same cell surface. This suggests that chicken histocompatibility antigens, may not be located close to the Con A receptor sites. Since the specific receptors for Con A are known to be associated with some of the polysaccharides of the cell membrane (Goldstein et al., 1965; Leon, 1967), these polysaccharides are probably not the B alloantigens. The cell adherence induced by Con A is not due to adsorption of the mitogen to the glass or plastic surfaces, since treatment of these surfaces with Con A does not induce cell adherence in the presence of serum, and the continued presence of Con A is required for cell adherence. No enhancement of adherence has been detected with Con A-treated tubes. The continued presence of Con A and PHA in tissue cultures is required for the continued stimulation of cell transformation (Jones, 1973; Lindahl-Kiessling, 1972; Powell and Leon, 1970). This suggests that Con A mediated transformation and Con A mediated adherence may involve an initially similar alteration of the cell membrane or cell activity.





## Discussion

Con A binds strongly to the plasma membrane of lymphocytes, and this binding is believed to induce blast transformation (Leon and Powell, 1968; Powell and Leon, 1970). While this plant lectin does not induce agglutination in normal cells, cells transformed by oncogenic viruses are agglutinated by the lectin. The results of my studies show that normal chicken lymphocytes are not agglutinated by Con A, although it is able to induce cell adherence in the presence of serum or other proteins. Cell adherence is therefore a manifestation of the interaction of surface receptors on the cell membrane with the specific agents. This supports my interpretation of allofixation as probably representing the interaction of surface alloantigens with specific allo-antibodies. The possibility that the specific adherence of cells to surfaces is a result of the interaction of cell surface receptors with their specific reagents is of great significance in the isolation and separation of functionally different though morphologically identical lymphoid populations. Specific receptors for hormones such as insulin, glucagon (Cuatrecasas, 1971) and corticosterone (Munck and Brinck-Johnson, 1968; Schaumburg and Bojesen, 1968; Schaumburg and Crone, 1971) and for drugs and other biologically active molecules such as PHA (Allan et al., 1971), Con A (Nicolson and Singer, 1971) and acetylcholine (Changeux et al., 1971; Miledi et al., 1971) have been demonstrated at the cell surface. Isolation of those cells which contain specific receptors from the heterogenous lymphoid populations is important for an understanding of the functions and nature of responses of these cells. Further propagation in tissue culture of cells





containing specific receptors may provide us with material for future experimentation.

The adherence-inducing component of the lectins is probably associated with the agglutinin rather than the mitogenic fraction of the proteins, since adherence of cells does not occur with pokeweed mitogen. Pokeweed mitogen is known to induce transformation of cells, although it lacks hemagglutinating and leukoagglutinating activity (Farnes et al., 1964). This implicates the leukoagglutinins as the active components in the induction of cell adherence. The difference in agglutinating activity of Con A and PHA is also detected by cell adherence. PHA is known to agglutinate both normal and transformed cells and Con A is known to agglutinate only transformed cells (Aub et al., 1965; Burger and Goldberg, 1967; Inbar and Sachs, 1969; Moscona, 1971; Steck and Wallach, 1969; Takahashi et al., 1967). The difference in agglutination seen with these two mitogens may be related to the valency and sizes of the molecules, as has been postulated for human alloagglutinins (Coombs and Gell, 1963). Con A is probably similar to the human incomplete alloagglutinins or 7S antibodies, while PHA agglutinin(s) may be similar to the complete alloagglutinins or 19S antibodies. The isolation and separation of agglutinins and the mitogenic factor from PHA has been achieved (Allen et al., 1969; Rivera and Meuller, 1966), although the separation of two such components from Con A has not been accomplished. The presence of two or more agglutinins in PHA proteins has been described (Adelson et al., 1965; Borjeson et al., 1960; Nordman et al., 1964; Sengar et al., 1970; Tunis, 1964). One of the PHA agglutinins is a potent hemagglutinin and displays the ability to



precipitate serum proteins nonspecifically. The other agglutinin is a weak hemagglutinin which agglutinates leukocytes and stimulates RNA synthesis. This agglutinin is difficult to separate from the mitogenic factor(s) and lacks the ability to precipitate serum proteins (Rivera and Mueller, 1966; Yachnin and Svenson, 1972). This may explain the association of lymphocyte agglutination with cell adherence when PHA is used, since the agglutinins of PHA are very heterogenous and some may have a high valency and tend to agglutinate rather than induce cell adherence. A low valency of Con A molecules may be used to explain the occurrence of cell adherence when Con A alone is used.

The results have shown that adherence of lymphocytes induced by Con A is temperature-sensitive and independent of pH. Agglutination and activation of cell transformation by Con A and PHA have been reported to be associated with a temperature-sensitive activity (Inbar et al., 1971, 1973), although the binding of Con A or PHA to cell surfaces is independent of temperature (Barat and Avrames, 1973; Inbar et al., 1971; Sällström and Alm, 1972). This temperature-sensitivity may be a metabolic activity and/or nonmetabolic, temperature-dependent structural rearrangement. The metabolic inhibitors iodoacetamide and sodium cyanide inhibit the cell adherence induced by Con A. It is known that binding of Con A to the cell surface is not inhibited by these two metabolic inhibitors, whereas the "cap formation" on the cell surface is inhibited (Sällström and Alm, 1972). This suggests that cell adherence to surfaces probably requires the "cap formation." "Cap formation" is sensitive to change of temperature, decreasing at low temperature (Sällström and Alm, 1972; Taylor et al., 1971).





Inhibition of "cap formation" thus coincides with the loss of cell adherence at low temperature. This further suggests the importance of "cap formation" in cell adherence. The effect of cyanide on "cap formation" is reversible while the effect of iodoacetamide on "cap formation" is not (Sällström and Alm, 1972). This could explain the difference in inhibition of cell adherence by cyanide and iodoacetamide. Iodoacetamide completely inhibits cell adherence whereas only partial inhibition is obtained by the same concentration of cyanide. Inhibition of "cap formation" by the metabolic inhibitors sodium azide and dinitrophenol has been reported by Taylor et al. (1971). "Cap formation" has also been observed with phytomitogens such as PHA (Osunkoya et al., 1970) and Con A (Smith and Hollers, 1970; Sällström and Alm, 1972). Thus it appears that the interaction of Con A with cell surface receptors is a non-metabolic process and "cap formation" is a metabolically dependent active process. However, the association of cell adherence with "cap formation" is contradicted by observations of adherence and "cap formation" in thymus and bursa cells: adherence of bursa cells was observed with the adherence test whereas "cap formation" is very rare in bursa cells (Kincade et al., 1971; Sällström and Alm, 1972). Thus "cap formation" may not be the mechanism involved in the adherence or fixation of cells.



Table 1. The Specificity of Anti-B<sub>14</sub> Serum produced in a  $\underline{B}^{13}/\underline{B}^{13}$  Recipient and Absorbed with  $\underline{B}^2/\underline{B}^2$  and  $\underline{B}^1/\underline{B}^1$  Erythrocytes

Genotypes of Lymphocytes	Bird Number	One Hour		Two Hours	
		1:4	1:8	1:4	1:8
		Percent Adhering Lymphocytes			
$\underline{B}^1/\underline{B}^1$	24390	10.6	3.3	6.4	5.4
	1268	-5.5	1.8	8.3	10.8
	867	-6.0	16.9	-14.3	-3.1
	1121	7.6	2.4	13.8	0.7
$\underline{B}^2/\underline{B}^2$	942	2.2	1.9	8.5	-5.0
	942	7.5	-3.5	8.2	-10.1
	1066	2.7	-9.3	1.1	6.6
	1066	-7.2	-3.0	-3.2	10.6
	24387	16.7	0.0	14.0	0.0
	605	-14.8	0.0	4.2	0.0
$\underline{B}^{13}/\underline{B}^{13}$	572	7.6	-3.3	15.2	-7.1
	1119	2.5	-4.5	3.4	-6.7
	1100	1.6	-13.5	-4.8	2.7
	1347	10.4	-2.9	0.6	-9.9
$\underline{B}^-/\underline{B}^{14}$	1289	57.1	22.6	78.1	57.3
	1057	76.6	65.4	87.5	88.4
	1223	75.3	73.9	93.3	91.9
	24378	65.8	67.0	91.8	94.8
	24377	61.2	65.3	83.6	85.9
	584	64.9	66.1	85.8	81.3
	24363	70.5	80.2	91.2	92.2
$\underline{B}^{14}/\underline{B}^{14}$	1284	83.4	75.8	97.3	98.2
	1083	69.9	73.6	91.3	95.9
	956	68.1	69.2	91.7	96.3
	24366	72.7	61.8	92.9	94.0
	24364	64.7	55.2	90.3	83.4
	24364	87.9	76.8	96.6	91.5
	24364	76.7	84.8	95.4	97.4



Table 2. Graft-versus Host Competence of Adhering and Non-Adhering Lymphocytes

Cell Suspension Medium	GVHR Competence of Lymphocytes: Number of CAM Pocks	
	Non-Adhering Cells	Adhering Cells
HBSS	2	1404
HBSS + FP	775	8
HBSS + FP + AS	181	1465

HBSS = Hanks' balanced salt solution, FP = fresh plasma, AS = antiserum

Table 3. Allofixation by Antiserum Coated Dishes

Treatment	Percent Adhering Erythrocytes
Untreated Dish + 10% FP	-2%
AS Treated Dish + 10% FP	85%
AS Treated Dish + 10% AGS	78%
AS Dish Treated with Saline	48%
AS Dish Treated with FP	11%
AS Dish Treated with Pepsin	40%
AS Dish Treated with Papain	11%

AS = antiserum, FP = fresh plasma, AGS = agamma serum





Table 4. Allofixation and Hemagglutination Titers of Primary, Secondary and Tertiary Antisera

Genotypes	Donors/ Recipients	Specificity of AS	AF Titer	HA Titer	AF/HA
$\underline{B}^2/\underline{B}^2$ to $\underline{B}^1/\underline{B}^{14}$	1226/26212(1) 619/26212(2) 619/26212(3)	B2(21)(15) B2(21)(15) (13)	<8 256 1024	16 256 128	<0.50 1.00 8.00
$\underline{B}^{14}/\underline{B}^{14}$ to $\underline{B}^1/\underline{B}^2$	28464/1260(1) 28464/1260(2) 28464/1260(3)	B14(15) B14(13)(15) (21)	32 128 2048	32 <sup>+</sup> 64 128 128	0.80 1.00 16.00
$\underline{B}^{14}/\underline{B}^{14}$ to $\underline{B}^2/\underline{B}^2$	28464/28482(1) 28464/28482(2) 28464/28482(3)	B14(1)(13) (15)(21) (15)(21)	<8 128 512	32 <sup>+</sup> 64 128 64	<0.15 1.00 8.00
$\underline{B}^2/\underline{B}^2$ to $\underline{B}^1/\underline{B}^{14}$	1226/26189(1) 619/26189(2) 619/26189(3)	B2(15) B2(15)(21) (13)	<4 32 256	16 32 64	<0.25 1.00 4.00
$\underline{B}^{14}/\underline{B}^{14}$ to $\underline{B}^2/\underline{B}^2$	1276/942(1) 1276/942(2)	B14(1)(13) (15) B14(1)(13) (15)(21)	32 512	64 128	0.50 4.00
$\underline{B}^{13}/\underline{B}^{13}$ to $\underline{B}^1/\underline{B}^{14}$	1277/1057(1) 1277/1057(2)	B13* B13*	32 1024	8 8	4.00 128
$\underline{B}^{13}/\underline{B}^{13}$ to $\underline{B}^2/\underline{B}^{14}$	1268/288(1) 1268/288(2)	B13* B13*	4 64	4 8	1.00 8.00
$\underline{B}^{21}/\underline{B}^{21}$ to $\underline{B}^1/\underline{B}^1$	1282/867(1) 1282/867(2)	B21(2)(13) (14)(15)	512 512	128 128	4.00 4.00

AF = allofixation, HA = hemagglutination, AS = antiserum,  
 (1)(2)(3) of second column = primary, secondary and tertiary antisera  
 respectively,  
 Number in parentheses of third column represents the HA cross reactions  
 of AS,  
 < = less than, \* indicates specific AS, titer is represented by reciprocal  
 of the dilutions of AS.



Table 5a. Strength of Cross Reactions of Unabsorbed Anti-B<sub>2</sub> Serum  
Produced in a  $\underline{B}^1/\underline{B}^{14}$  Bird.

Genotypes	Primary		Secondary		Tertiary	
	HA	AF	HA	AF	HA	AF
$\underline{B}^1/\underline{B}^1$	<1:2	<1:8	<1:2	<1:8	<1:2	<1:8
$\underline{B}^2/\underline{B}^2$	1:16	<1:8	1:256	1:256	1:128	1:1024
$\underline{B}^{13}/\underline{B}^{13}$	<1:4	<1:8	1:16	1:64+	1:32	1:128+
$\underline{B}^{13}/\underline{B}^{15}$	<1:4	<1:8	1:128	1:32	1:64	1:64+
$\underline{B}^{15}/\underline{B}^{15}$	<1:4	<1:8	1:32+	<1:8	1:64	1:8
$\underline{B}^{14}/\underline{B}^{14}$	<1:2	<1:8	<1:2	<1:8	<1:2	<1:8
$\underline{B}^{21}/\underline{B}^{21}$	1:8	<1:8	1:16+	1:8	1:32+	1:32

HA = hemagglutination, AF = allofixation

Table 5b. Strength of Cross Reactions of Unabsorbed Anti-B<sub>21</sub> Serum  
Produced in a  $\underline{B}^1/\underline{B}^1$  Bird.

Genotypes	Secondary		Tertiary	
	HA	AF	HA	AF
$\underline{B}^1/\underline{B}^1$	<1:2	<1:8	<1:2	<1:8
$\underline{B}^2/\underline{B}^2$	1:64	1:128	1:64	1:128+
$\underline{B}^{13}/\underline{B}^{15}$	1:128	1:512	1:64	1:512
$\underline{B}^{14}/\underline{B}^{14}$	1:16+	1:128	1:32	1:256
$\underline{B}^{21}/\underline{B}^{21}$	1:128	1:512	1:128	1:512

HA = hemagglutination, AF = allofixation





Table 6. Cross Reactions of Unabsorbed Anti-B<sub>14</sub> Serum Produced in a B<sup>2</sup>/B<sup>2</sup> Bird.

Genotypes	Bird Number	Antiserum Dilutions			
		1:4	1:8	1:16	1:32
		Percent Adhering Lymphocytes			
<u>B<sup>1</sup></u> / <u>B<sup>1</sup></u>	1264	59.63	68.01	71.43	50.81
	1121	64.31	84.17	93.77	91.34
<u>B<sup>2</sup></u> / <u>B<sup>2</sup></u>	728	-2.79	8.31	15.34	1.77
	1066	12.74	15.41	-7.91	1.48
<u>B<sup>13</sup></u> / <u>B<sup>13</sup></u>	1100	36.01	85.19	94.13	78.72
	1277	63.26	95.82	94.32	94.12
<u>B<sup>14</sup></u> / <u>B<sup>14</sup></u>	1284	28.12	68.58	74.26	69.71
	956	65.12	78.81	77.51	57.65



Table 7. Cross Reactions of Anti-B<sub>14</sub> Serum Produced in a  $\underline{B}^2/\underline{B}^2$  Bird and Absorbed with  $\underline{B}^1/\underline{B}^1$  and  $\underline{B}^{13}/\underline{B}^{13}$  Erythrocytes.

Genotypes	Bird Number	Antiserum Dilutions			HA titer
		1:4	1:8	1:16	
		Percent Adhering Lymphocytes			
$\underline{B}^1/\underline{B}^1$	1121	66.14	65.53	53.44	<1:4
	867	33.09	45.72	23.45	<1:4
	1268	45.35	57.51	23.45	<1:4
	1121	70.00	50.85	14.55	<1:4
	1264	48.97	78.57	73.40	<1:4
$\underline{B}^2/\underline{B}^2$	942	22.47	19.78	1.55	<1:4
	942	3.38	44.91	7.78	<1:4
	942	12.20	2.35	-7.63	<1:4
$\underline{B}^{13}/\underline{B}^{13}$	1347	13.60	18.34	7.67	<1:4
	1100	26.74	25.57	31.90	<1:4
	1119	33.47	43.14	23.20	<1:4
	1347	27.10	19.26	-0.41	<1:4
	1100	11.43	29.41	13.83	<1:4
	1277	19.86	20.45	29.17	<1:4
$\underline{B}^1/\underline{B}^{14}$	1057	35.71	66.96	51.49	1:32
	1289	43.90	59.70	58.31	1:32
$\underline{B}^{14}/\underline{B}^{14}$	1284	75.53	73.40	46.28	1:32
	1284	68.60	75.43	79.15	1:64
	956	49.65	43.87	47.78	1:32
	1284	85.50	88.44	92.83	1:64



Table 8. Cross Reactions of Anti-B<sub>14</sub> Serum Produced in a B<sup>13</sup>/B<sup>13</sup> Bird and Absorbed with B<sup>1</sup>/B<sup>1</sup> and B<sup>2</sup>/B<sup>2</sup> Erythrocytes.

Genotypes	Bird Number	Antiserum Dilutions			HA Titer
		1:4	1:8	1:16	
		Percent Adhering Lymphocytes			
<u>B</u> <sup>1</sup> / <u>B</u> <sup>1</sup>	1121	13.82	0.88	-12.19	1:4 <sup>+</sup> <sub>8</sub>
	867	12.00	-14.28	-3.08	<1:4
	1268	5.37	8.31	10.80	<1:4
	1121	-9.97	6.23	6.80	1:4 <sup>+</sup> <sub>8</sub>
<u>B</u> <sup>2</sup> / <u>B</u> <sup>2</sup>	942	8.81	-10.12	7.03	1:4 <sup>+</sup> <sub>8</sub>
	942	5.92	8.49	-5.02	<1:4
	942	6.96	2.73	4.56	<1:4
<u>B</u> <sup>13</sup> / <u>B</u> <sup>13</sup>	1347	0.65	-9.92	0.79	<1:4
	1100	6.12	-4.79	2.69	<1:4
	1119	13.33	3.36	-6.70	<1:4
	1347	-9.30	1.36	5.80	<1:4
<u>B</u> <sup>-</sup> / <u>B</u> <sup>14</sup>	1057	71.18	87.47	88.39	>1:64
	1289	57.58	78.11	57.35	1:32
<u>B</u> <sup>14</sup> / <u>B</u> <sup>14</sup>	1284	97.29	98.22	93.77	1:64
	1284	90.48	62.75	-8.08	>1:64

HA = hemagglutination





Table 9. Allofixation and Hemagglutination Titers of Untreated and Mercaptoethanol-Treated Antisera.

Antisera	Specificity	Untreated		ME-Treated		AF ME/Control	HA
		AF	HA	AF	HA		
Primary	Anti-B <sub>14</sub>	1:32	1:16+	<1:4	1:4+	≈0	0.25
		1:32	1:64	<1:16	ND	≈0	ND
		1:32	1:64	<1:16	ND	≈0	ND
	Anti-B <sub>13</sub>	1:32	1:64	<1:4	1:32	≈0	0.50
		<1:4	1:32	<1:4	1:16+	≈0	0.75
		1:32+	1:8	<1:8	<1:4	≈0	≈0.00
Hyper- Immune	Anti-B <sub>14</sub>	1:2048	1:128	1:2048	1:64	1.00	0.50
		1:512+	1:128	1:512+	1:64+	1.00	0.75
		1:256+	1:128	1:128	1:64+	≈1.00	0.75
		1:128	1:64	1:128	1:32+	1.00	0.75
	Anti-B <sub>13</sub>	1:256+	1:128	1:256+	1:64+	≈1.00	0.75
		1:512	1:64	1:512	1:32+	1.00	0.75
		1:1024+	1:8	1:1024	<1:4	0.75	≈0.00
		1:64+	1:8	1:64	<1:4	0.75	≈0.00

AF = allofixation, HA = hemagglutination, ND = not done,  
ME = mercaptoethanol, ≈ = approximately equal to.



Table 10. Allofixation of Untreated, Heat Inactivated Antiserum and Salt-Precipitated Globulins.

Diluents	Na <sub>2</sub> SO <sub>4</sub> ppt	Percent Adhering Lymphocytes			
		Globulins		Antiserum	
		Untreated	Heated	Untreated	Heated
HBSS	36.2		3.3	95.9	1.8
BBS	59.2		1.4	92.0	4.1
FP	96.4		95.2	95.7	83.5
Heated Plasma	9.9		27.6	64.6	16.6

HBSS = Hanks' balanced salt solution, BBS = borate buffered saline, FP = fresh plasma.

Table 11. Allofixation and Hemagglutination Titers of Partially Purified IgM and IgG Globulins.

	Control		ME-Treated		AF <sub>me</sub> / AF <sub>c</sub>	HA <sub>me</sub> / HA <sub>c</sub>
	AF	HA	AF	HA		
IgM	1:32	1:8+	1:16	1:4+	0.50	0.50
	1:32	1:8+	1:16	1:4+	0.50	0.50
	1:32	1:8	1:8	1:4+	?	0.75
IgG	1:512	1:32+	1:256+	1:16+	0.75	0.50
	1:512	1:32	1:256+	1:16+	0.75	0.75
	1:512	1:32	1:512	1:32	0.75	0.75

AF = allofixation, HA = hemagglutination, ME = mercaptoethanol

HA<sub>me</sub> or AF<sub>me</sub> = hemagglutination or allofixation titer of ME-treated immunoglobulin.

HA<sub>c</sub> or AF<sub>c</sub> = hemagglutination or allofixation titer of untreated immunoglobulin.





Table 12. Adherence of Mouse Lymph Node Cells with Chicken Anti—Mouse Red Cell— Serum.

Treatments	% Adhering Lymph Node Cells				Remarks
	Balb/C L.N.	CBA L.N.	Mean		
AS + HBSS	85	95	90	90	Cytotoxicity
CFP + HBSS	16	23	19	19	
HAS + HBSS	58	69	60	62	Agglutination
HAS + CFP	43	47	39	43	?
HAS + MFP	33	33	18	28	Agglutination
MFP + HBSS	5	11	8	8	

AS = antiserum, HBSS = Hanks' balanced salt solution, CFP = fresh chicken plasma, MFP = fresh mouse plasma, HAS = heated antiserum, L.N. = lymph node, ? = cytotoxicity or agglutination.

Table 13 Concanavalin A-Induced Adherence of Chicken Lymphocytes from Different Genetic Lines.

Genotypes	Control	<u>Concentration of Con A</u>		
		50ug/ml	100ug/ml	200ug/ml
		% Adhering Lymphocytes		
<u>B</u> <sup>2</sup> / <u>B</u> <sup>2</sup>	10	63	76	78
<u>B</u> <sup>13</sup> / <u>B</u> <sup>13</sup>	-1	57	72	80
<u>B</u> <sup>14</sup> / <u>B</u> <sup>14</sup>	3	61	76	75
<u>B</u> <sup>15</sup> / <u>B</u> <sup>15</sup>	-1	56	69	77
<u>B</u> <sup>21</sup> / <u>B</u> <sup>21</sup>	-1	50	67	77



Table 14. Concanavalin A-Induced Cell Adherence of Thymus (T),  
Bursa (B) and Peripheral (P) Lymphocytes in Chicken.

First Experiment:								
Cells	Time	Concentration of Con A (ug/ml)						
		100	25	12.5	6.25	3.125	0.00	
Percent Adhering Lymphocytes								
P	1 Hr.	23	0	4	-7	5	-1	
	4 Hr.	67	-6	4	4	2	-3	
T	1 Hr.	24	2	-1	-11	-10	3	
	4 Hr.	84	9	-1	6	-10	3	
B	1 Hr.	62	37	12	17	6	-1	
	4 Hr.	89	60	27	36	18	10	
Second Experiment:								
Cells	Time	Concentration of Con A (ug/ml)						
		250	125	100	62.5	31.25	25.0	0.00
Percent Adhering Lymphocytes								
P	1 Hr.	35	20	15	8	-6	5	2
	2 Hr.	68	54	33	19	-7	4	2
T	1 Hr.	58	24	27	16	20	17	3
	2 Hr.	88	69	34	28	8	8	-18
B	1 Hr.	67	66	57	57	39	22	-12
	2 Hr.	79	84	72	73	63	30	2
Third Experiment:								
Cells	Time	Concentration of Con A (ug/ml)						
		300	200	100	50	25	10	0.00
Percent Adhering Lymphocytes								
P	1 Hr.	62	55	39	2	-6	-2	4
	2 Hr.	79	78	65	12	10	8	5
T	1 Hr.	60	65	34	2	3	2	10
	2 Hr.	75	88	67	12	6	4	18
B	1 Hr.	74	65	57	32	18	12	11
	2 Hr.	/	76	75	53	24	27	17







Figure 1. The effect of pH on aspecific adherence (HBSS), non-adherence in plasma (HBSS + FP), and allofixation (HBSS + FP + ANTI-SERUM). Lymphocytes of different genotypes were used for the test of aspecific adherence, non-adherence in plasma, whereas  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested with an unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  for allofixation. The standard errors for aspecific adherence and non-adherence in plasma are indicated by the parallel pairs of lines (—) flanking the means (—).

Figure 2. The effect of pH on allofixation of  $\underline{B}^2/\underline{B}^2$ ,  $\underline{B}^{14}/\underline{B}^{14}$ , and  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes with specific anti- $B_2$ , anti- $B_{14}$  and anti- $B_{15}$  respectively was run at room temperature for 2 hours. Four series of tests were performed on  $\underline{B}^2/\underline{B}^2$  and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes obtained from 4 different birds and 6 series of tests were run with  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes obtained from 6 different birds.

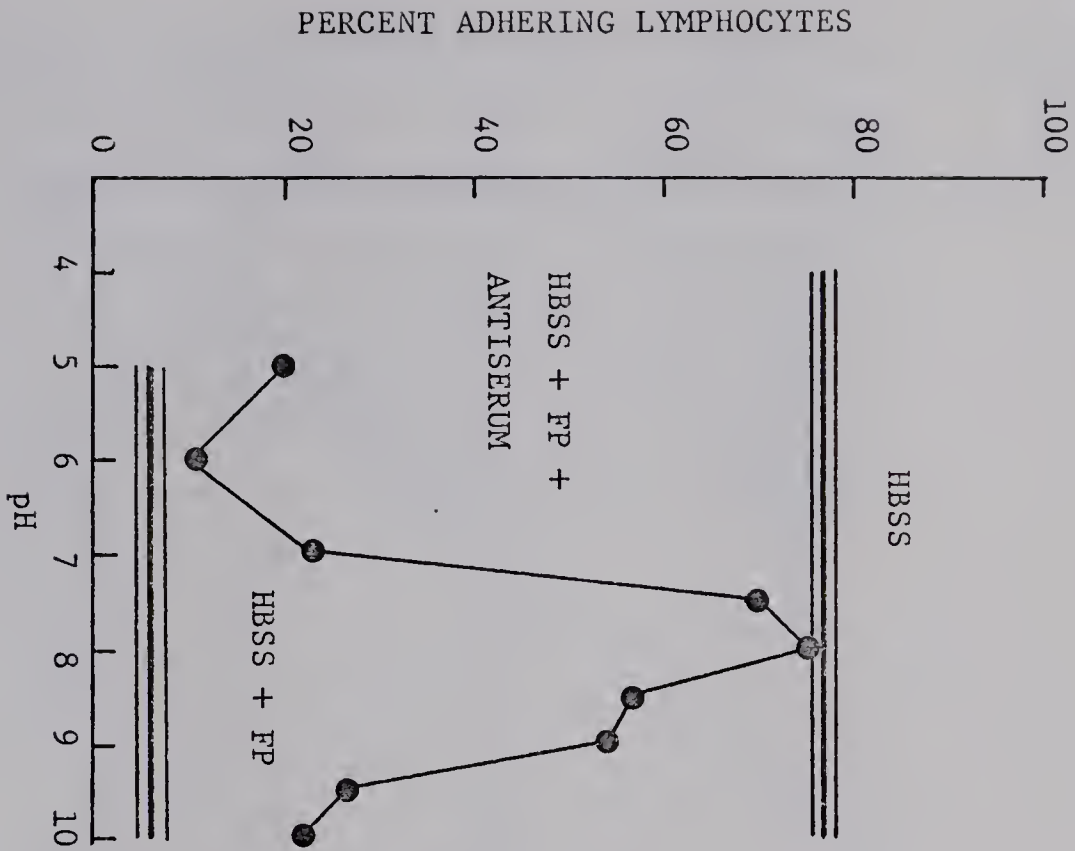


Figure 1

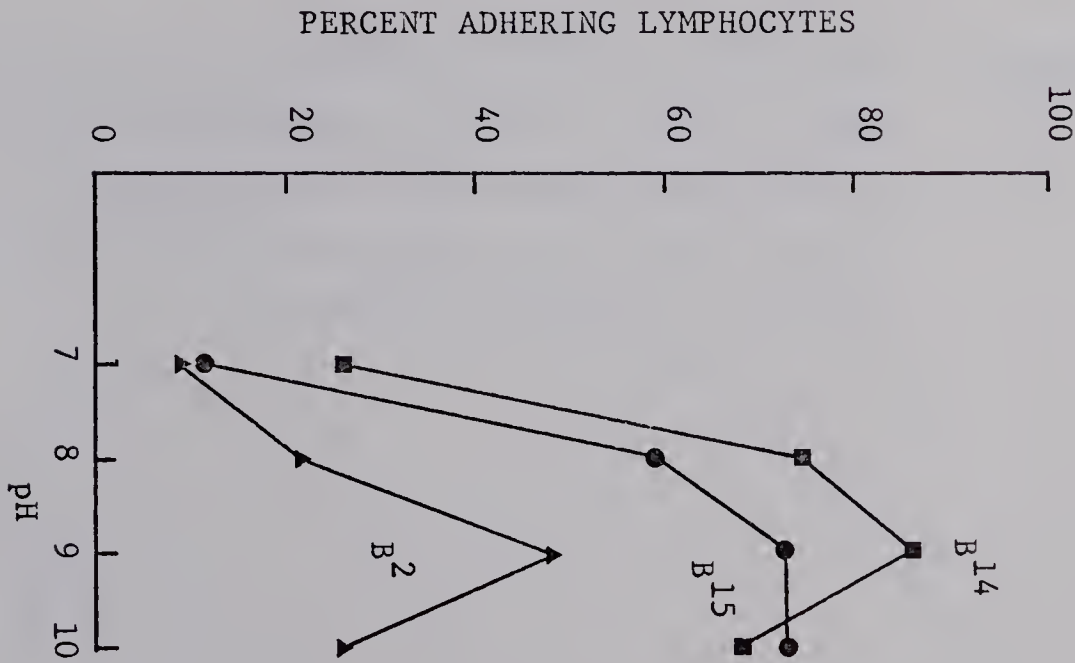


Figure 2





Figure 3. The effect of temperature on aspecific adherence (HBSS) and allofixation (AS + FP + HBSS).  $\underline{B}^2/\underline{B}^2$  lymphocytes were used for the test of aspecific adherence, whereas  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  for allofixation. The points indicate the means at each temperature. Both kinds of adherence are linear functions of temperature ( $p < 0.001$  for linear regression;  $p > 0.05$  for deviation from linearity).

Figure 4. The effect of time of incubation on aspecific adherence. Duplicate tests were run with  $\underline{B}^{13}/\underline{B}^{13}$  lymphocytes in HBSS at room temperature. Counts were taken at 15 minute intervals for the first hour of incubation and at 30 minute intervals for the second hour of incubation.



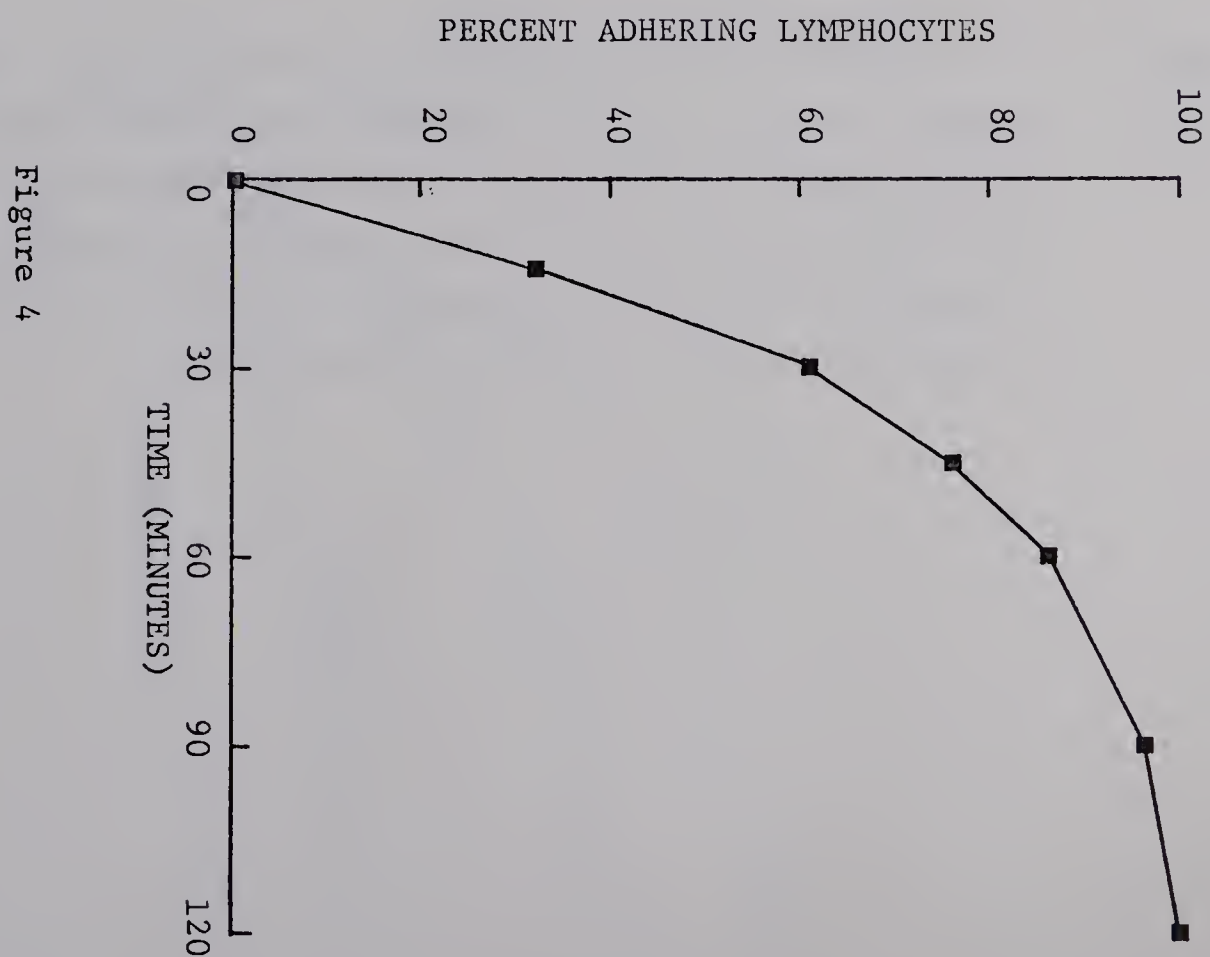
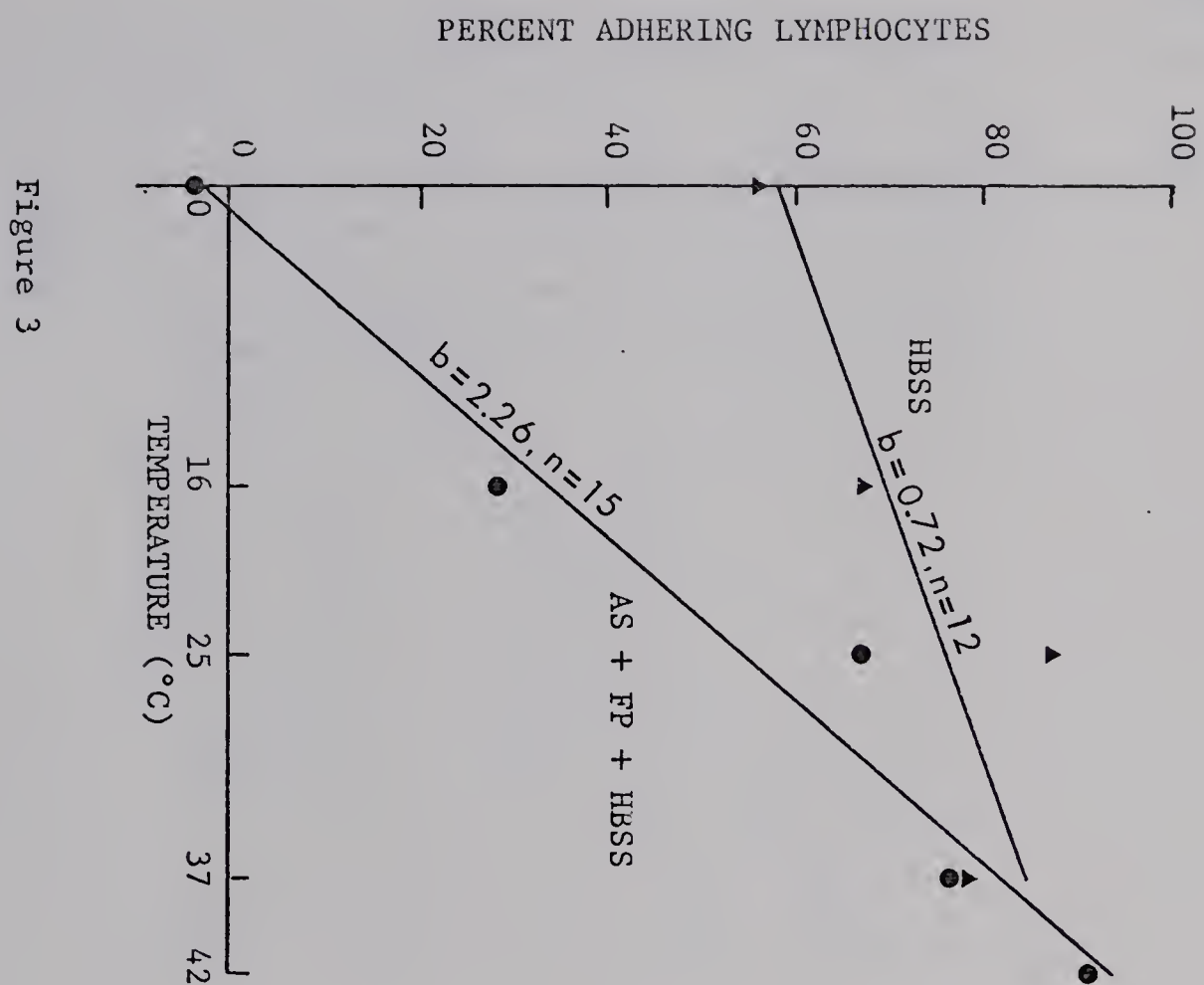






Figure 5. The effect of time of incubation on allofixation. Duplicate tests were run on  $\underline{B}^{13}/\underline{B}^{13}$ ,  $\underline{B}^{13}/\underline{B}^{15}$  and  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes with a specific anti- $B_{13}$  serum at room temperature and at pH 8.5. Counts were taken at 15 minute intervals for a period of 1 hour. The antiserum was tested at 1/8 dilution and the supplement of fresh chicken plasma.

Figure 6. The effect of time of incubation on allofixation. Duplicate tests were run on  $\underline{B}^{15}/\underline{B}^{15}$ ,  $\underline{B}^{15}/\underline{B}^{13}$  and  $\underline{B}^{13}/\underline{B}^{13}$  lymphocytes with a specific anti- $B_{15}$  serum at room temperature and at pH 8.5. Counts were taken at 15 minute intervals for a period of 1 hour. The antiserum was tested at 1/8 dilution and the supplement of fresh chicken plasma.

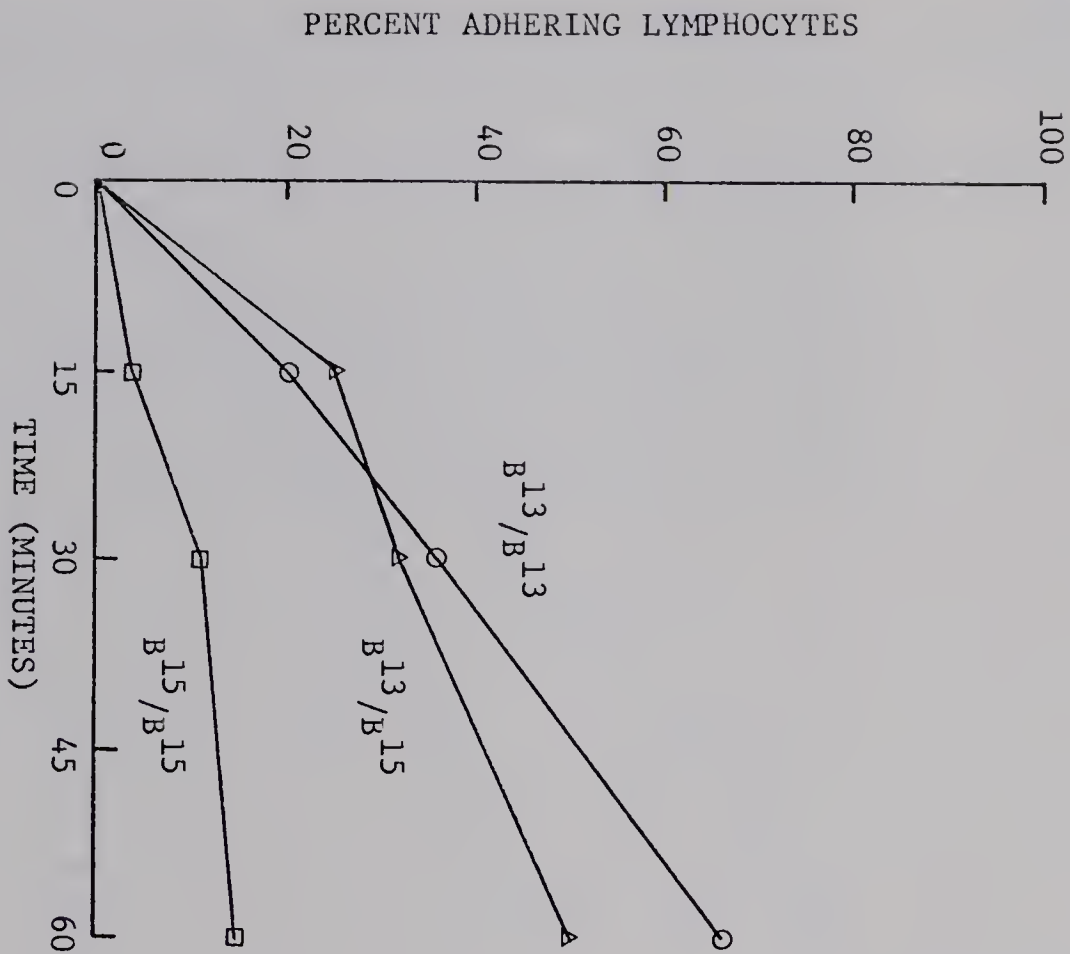


Figure 5

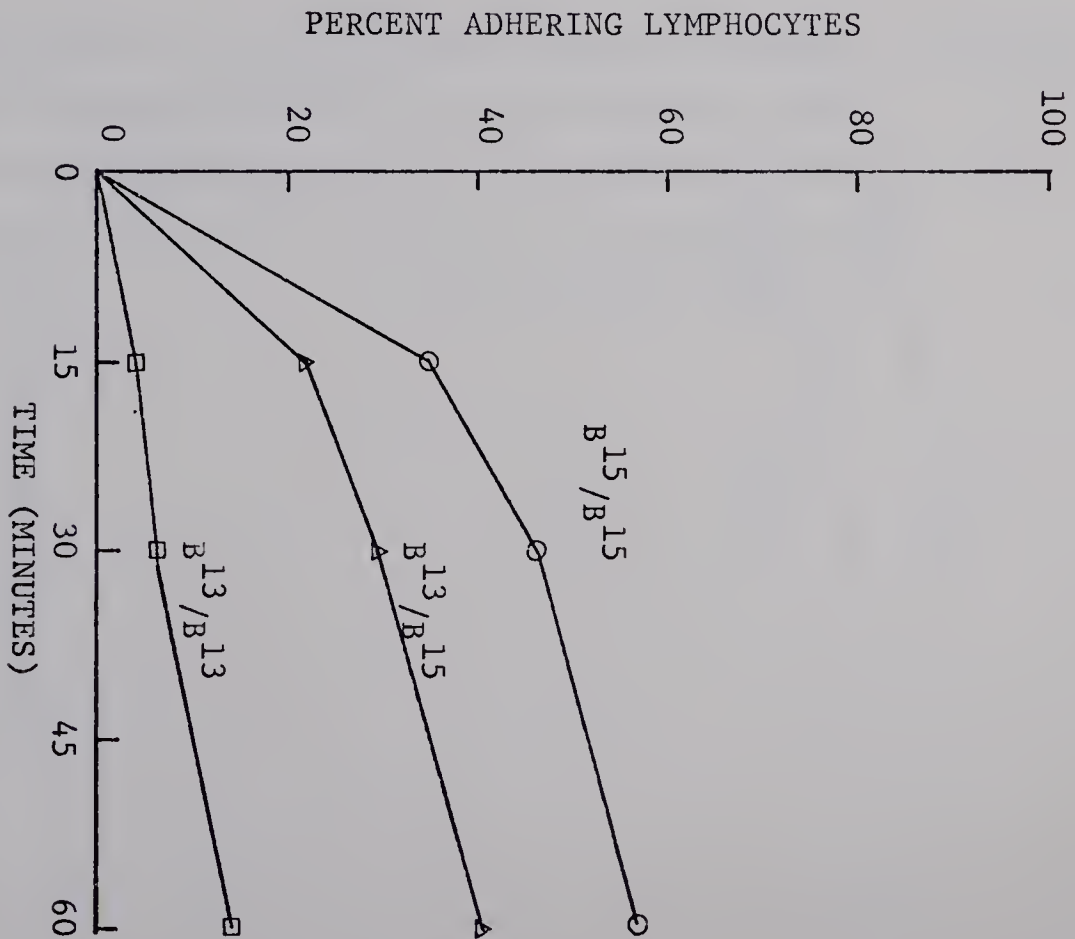


Figure 6







Figure 7. The effect of shaking on allofixation. Duplicate tests were run on  $\underline{B}^{13}/\underline{B}^{13}$  and  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes with anti- $B_{13}$  and anti- $B_{15}$  sera respectively. The tests were performed at 1/8 dilution of antisera and the supplement of fresh chicken plasma at room temperature and at pH 8.5. Counts were taken at 15 minute intervals for a period of 1 hour from the first tube ( $\square, O$ ), but the second, third and fourth tubes ( $\blacksquare, \odot$ ) were counted at 30, 45 and 60 minutes respectively.

Figure 8. The effect of cell concentration on allofixation. Duplicate tests were performed on  $\underline{B}^{13}/\underline{B}^{15}$  lymphocytes with a specific anti- $B_{13}$  serum at room temperature for 2 hours at pH 8.5. The antiserum was tested at 1/8 dilution with the supplement of fresh chicken plasma.

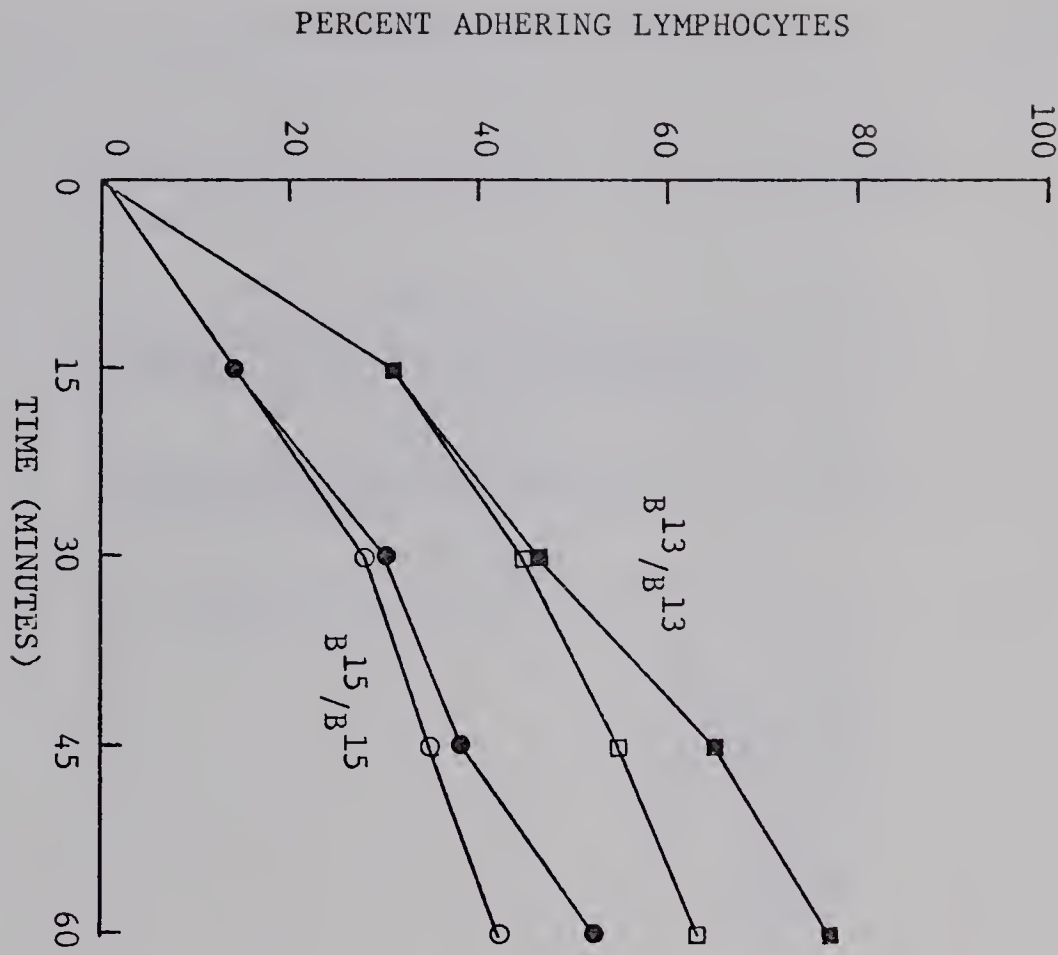


Figure 7

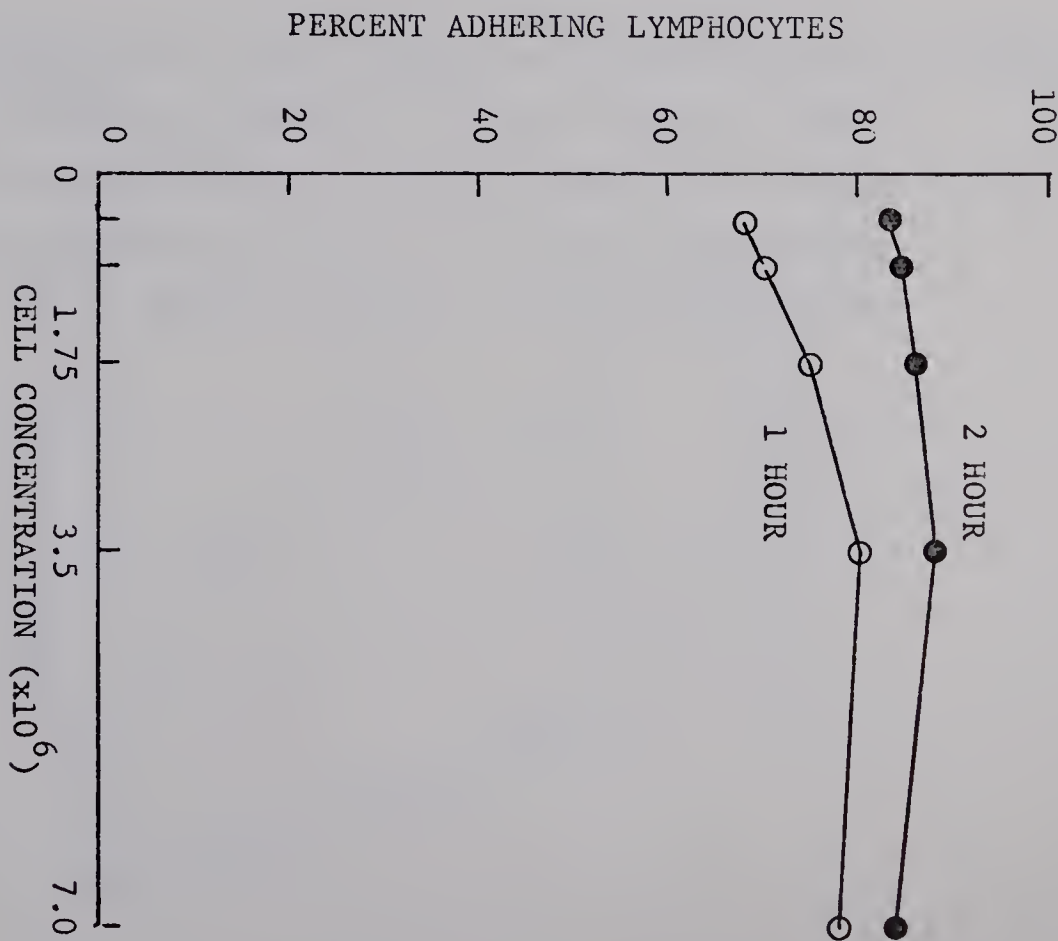


Figure 8







Figure 9. The effects on allofixation of treating the antiserum and omitting plasma.  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  after heating at 56°C for 30 minutes, dialyzed overnight or untreated. Three tests were done for each of nine experiments. The effects of altering the antiserum and omitting plasma were very highly significant ( $p < 0.001$  for both), but the omission of fresh plasma was the more inhibitory.

Figure 10. The allofixation of  $\underline{B}^2/\underline{B}^2$ ,  $\underline{B}^2/\underline{B}^{14}$ , and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$ . The tests were run at room temperature for 1 hour and each of the points represents the mean of 3 separate tests performed on lymphocytes obtained from 3 different birds.

Figure 9

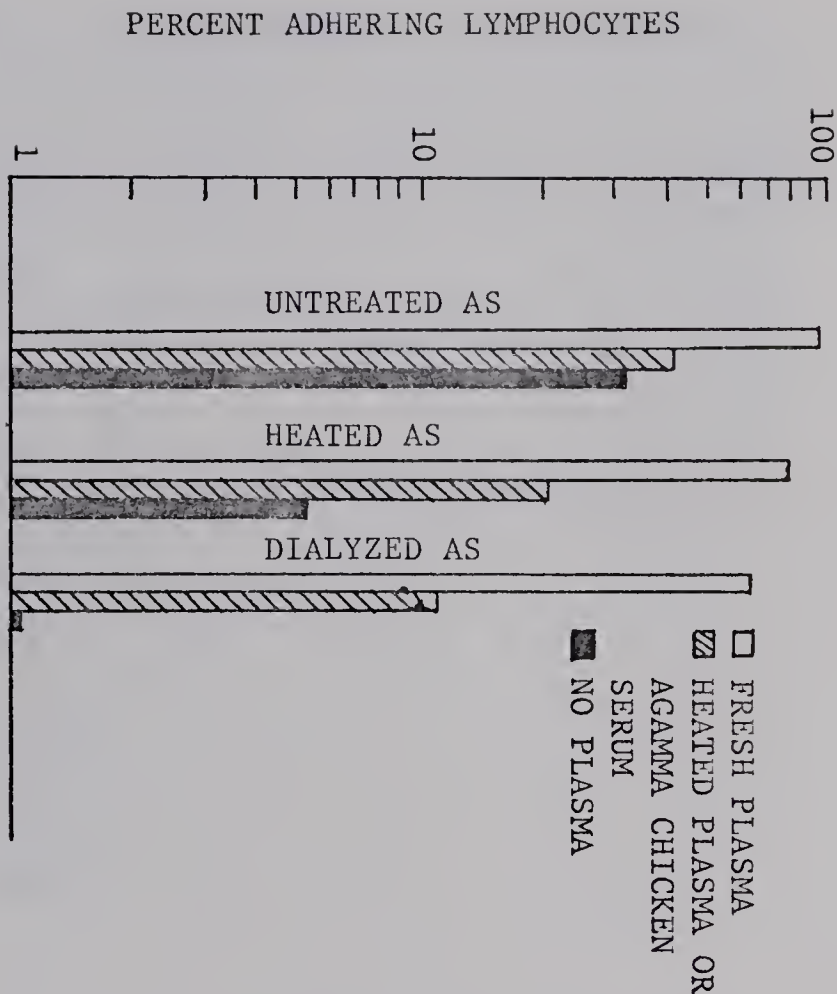


Figure 10

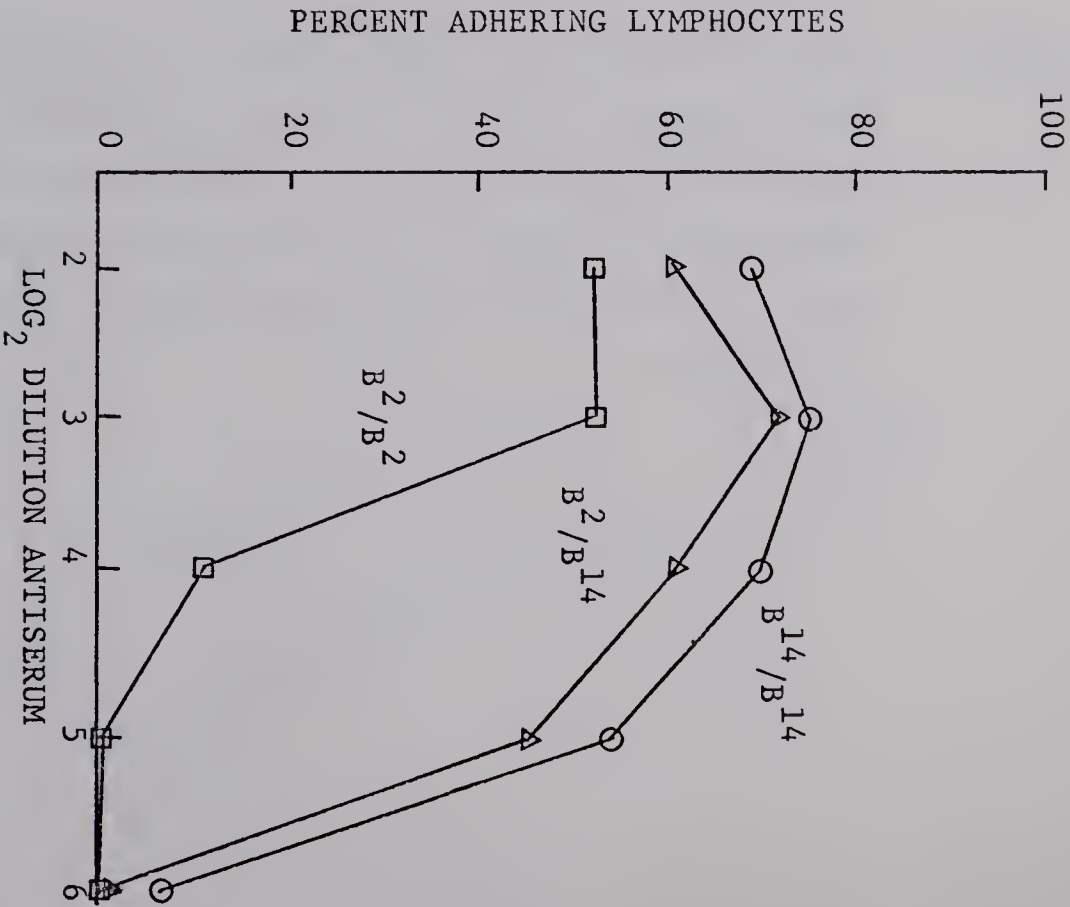








Figure 11. The allofixation of  $\underline{B}^2/\underline{B}^2$ ,  $\underline{B}^2/\underline{B}^{14}$ , and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  birds and absorbed with  $\underline{B}^1/\underline{B}^1$  and  $\underline{B}^2/\underline{B}^2$  erythrocytes. The tests were run at room temperature for 1 hour and each of the points represents the mean of 3 separate tests performed on lymphocytes obtained from 3 different birds.

Figure 12. The allofixation of  $\underline{B}^2/\underline{B}^2$ ,  $\underline{B}^2/\underline{B}^{14}$ , and  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with unabsorbed anti- $B_2$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$ . The tests were run at room temperature for 1 hour and each of the points represents the mean of 3 separate tests performed on lymphocytes obtained from 3 different birds of the same genotypes.

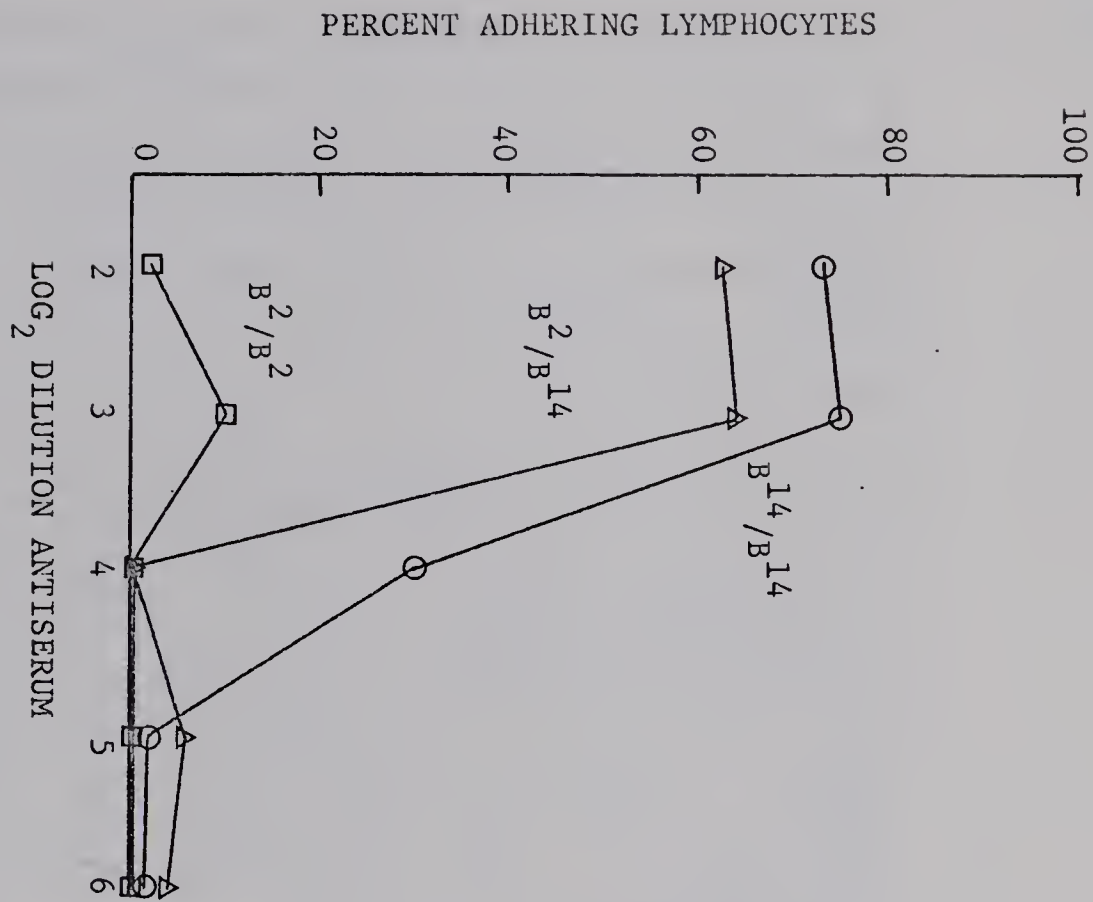


Figure 11

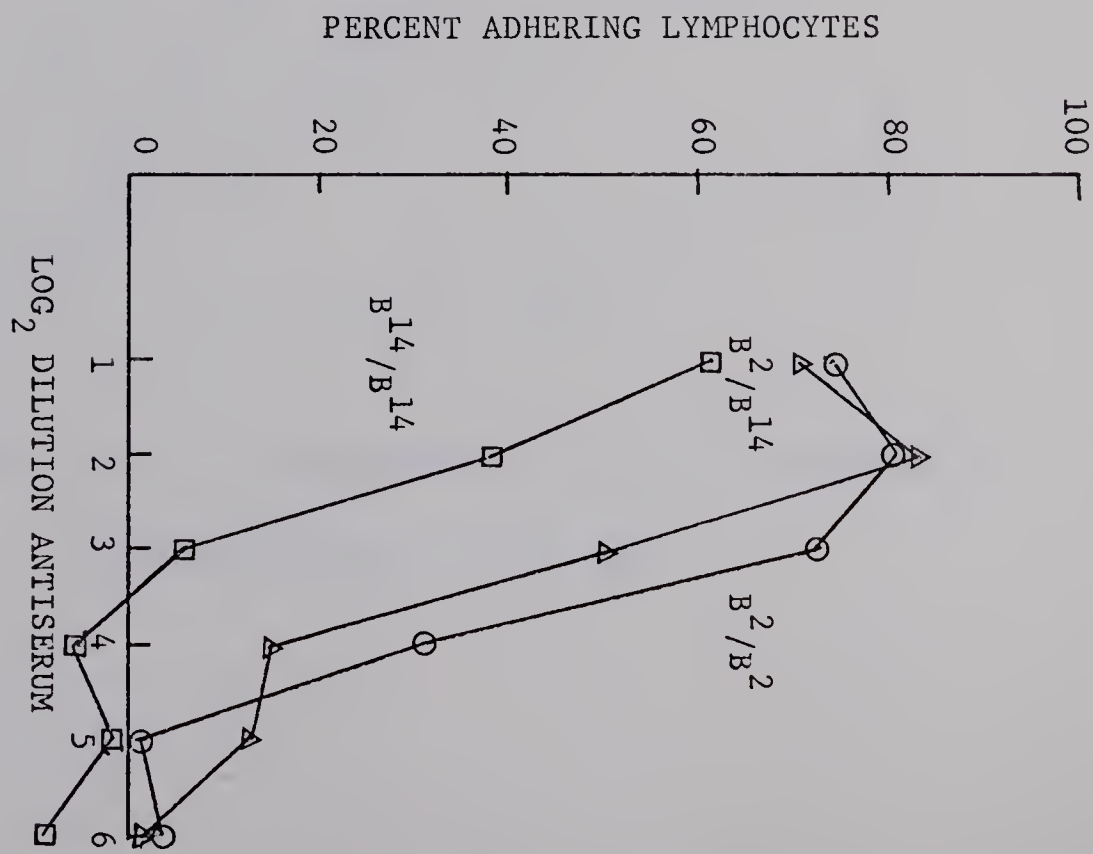


Figure 12





Figure 13. The allofixation of lymphocytes from A and B heterozygotes and homozygotes.  $\underline{A}^2/\underline{A}^2$ ,  $\underline{A}^2/\underline{A}^6$ , and  $\underline{A}^6/\underline{A}^6$  lymphocytes of different B genotypes were tested with specific anti- $\underline{A}_2$  and anti- $\underline{A}_6$ .  $\underline{B}^1/\underline{B}^1$ ,  $\underline{B}^2/\underline{B}^2$ ,  $\underline{B}^{13}/\underline{B}^{13}$ ,  $\underline{B}^{14}/\underline{B}^{14}$ ,  $\underline{B}^{15}/\underline{B}^{15}$  and  $\underline{B}^{21}/\underline{B}^{21}$  homozygotes and  $\underline{B}^1/\underline{B}^2$ ,  $\underline{B}^1/\underline{B}^{14}$ ,  $\underline{B}^2/\underline{B}^{14}$ ,  $\underline{B}^{13}/\underline{B}^{15}$ ,  $\underline{B}^{15}/\underline{B}^{21}$ , and  $\underline{B}^2/\underline{B}^{21}$  heterozygotes were tested with different specific antibodies (i.e. anti- $\underline{B}_1$ , anti- $\underline{B}_2$ , anti- $\underline{B}_{13}$ , anti- $\underline{B}_{14}$ , anti- $\underline{B}_{15}$ , and anti- $\underline{B}_{21}$ ). Additive gene dosage occurred for alleles  $\underline{A}^2$  ( $p$  for linear regression  $<0.001$ ; deviation from linearity  $>0.050$ ),  $\underline{A}^6$  ( $p < 0.001$  and  $p > 0.050$ ), and  $\underline{B}^2$  ( $p < 0.050$  and  $p > 0.050$ ), but not for  $\underline{B}^1$ ,  $\underline{B}^{13}$ ,  $\underline{B}^{14}$ , and  $\underline{B}^{15}$  at  $25^\circ\text{C}$ . The gene dosage effect of  $\underline{B}^{21}$  was uncertain due to difficulty in preparing a specific anti- $\underline{B}_{21}$  serum. The allofixation of  $\underline{B}^2/\underline{B}^{14}$  lymphocytes with anti- $\underline{B}_{14}$  was several times more rapid at  $25^\circ\text{C}$  than allofixation of these cells with anti- $\underline{B}_2$ .



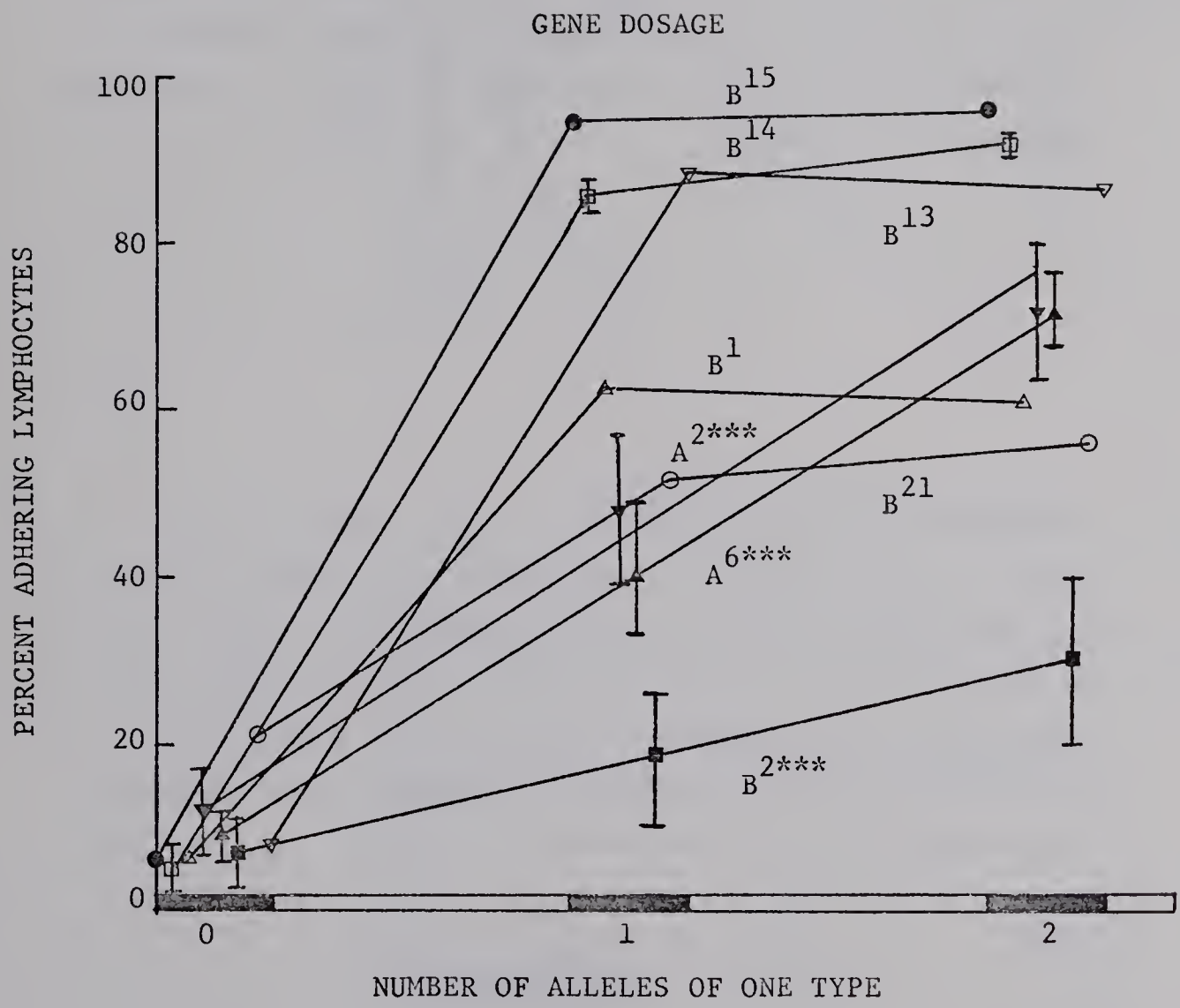


Figure 13





Figure 14. The allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  thymus (T), bursa (B) and peripheral (P) lymphocytes with specific anti- $B_{14}$  and anti- $B_{15}$  sera. Three series of tests were performed on a total of 12 birds and each of the points represents the mean of 3 separate tests on cells obtained from 3 different birds. The closed circles (●), triangles (▲) and squares (■) represent the allofixation of T, B and P cells with anti- $B_{14}$  and the open circles (○), triangles (Δ) and squares (□) represent the controls tested with anti- $B_{15}$ . Three different specific anti- $B_{14}$  sera and one specific anti- $B_{15}$  serum were employed in the tests.

Figure 15. The allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  thymus (T), bursa (B) and peripheral (P) lymphocytes with specific anti- $B_{14}$  and anti- $B_{15}$  sera. Lymphoid cells were obtained from a total of 12 different birds and each of the bars represents the mean of 12 different tests performed. The antisera were tested at 1/8 dilution with the supplement of fresh chicken plasma at room temperature for 2 hours and at pH 8.5.

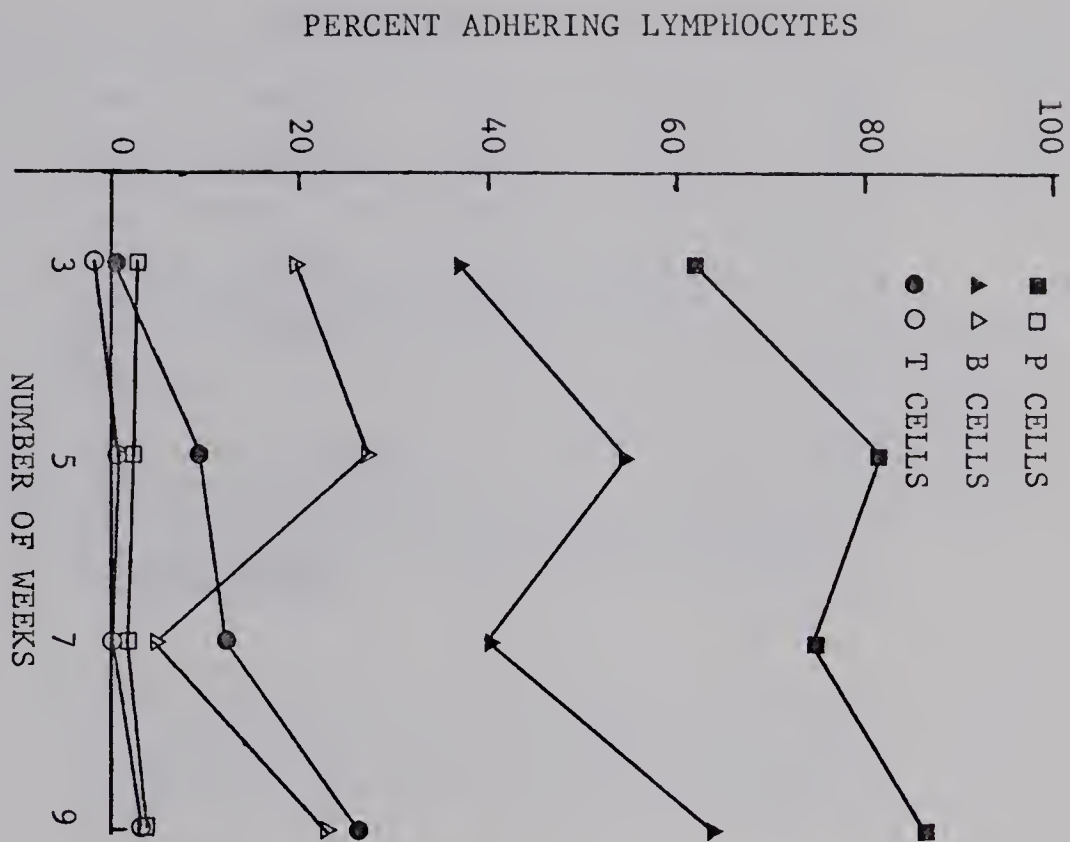


Figure 14

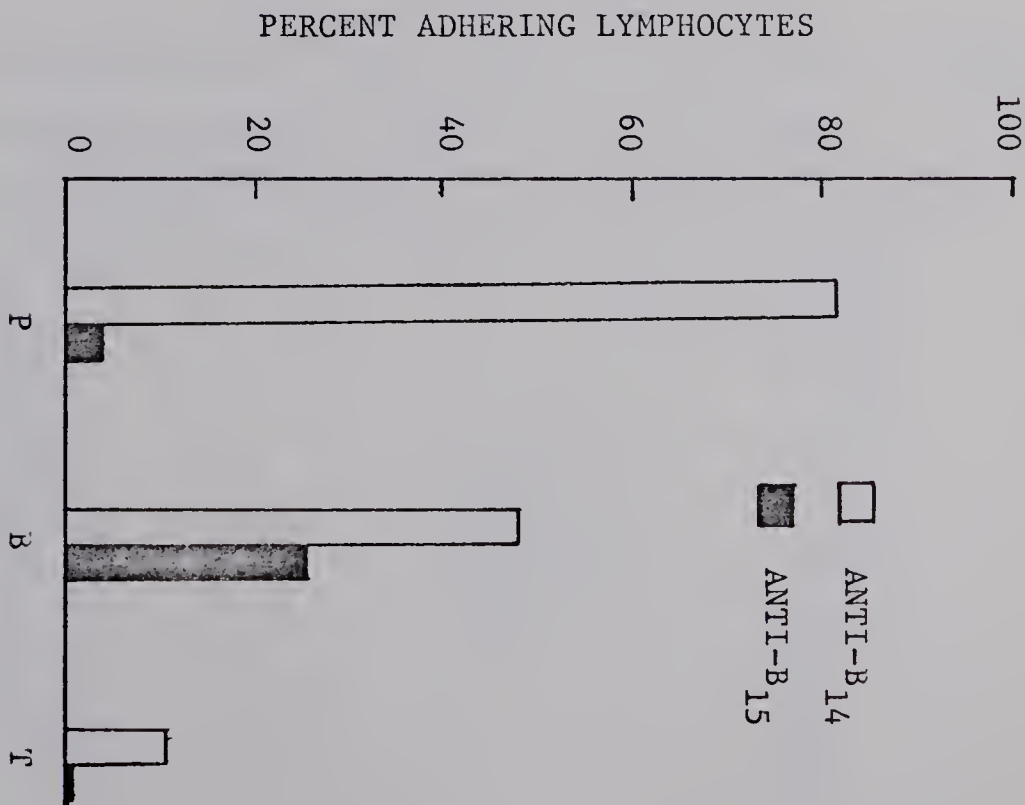


Figure 15







Figure 16. The allofixation of  $\underline{A}^6/\underline{A}^6$  thymus (T), bursa (B) and peripheral (P) lymphocytes with specific anti- $A_2$  and anti- $A_6$  sera. Lymphoid cells were obtained from a total of 8 different birds of which the  $\underline{B}$  genotypes were  $\underline{B}^2/\underline{B}^2$  (1),  $\underline{B}^2/\underline{B}^{14}$  (2) and  $\underline{B}^{14}/\underline{B}^{14}$  (5). The means of the 8 tests were plotted and the tests performed in HBSS plus fresh chicken plasma were used as control. The antisera were tested at 1/8 dilution and the supplement of fresh chicken plasma at room temperature with pH adjusted to 8.5 and incubated for 2 hours.

Figure 17. The effect of simple sugars on allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$ . Three series of tests were performed on lymphocytes obtained from 3 different birds, and each point represents the mean of the tests.

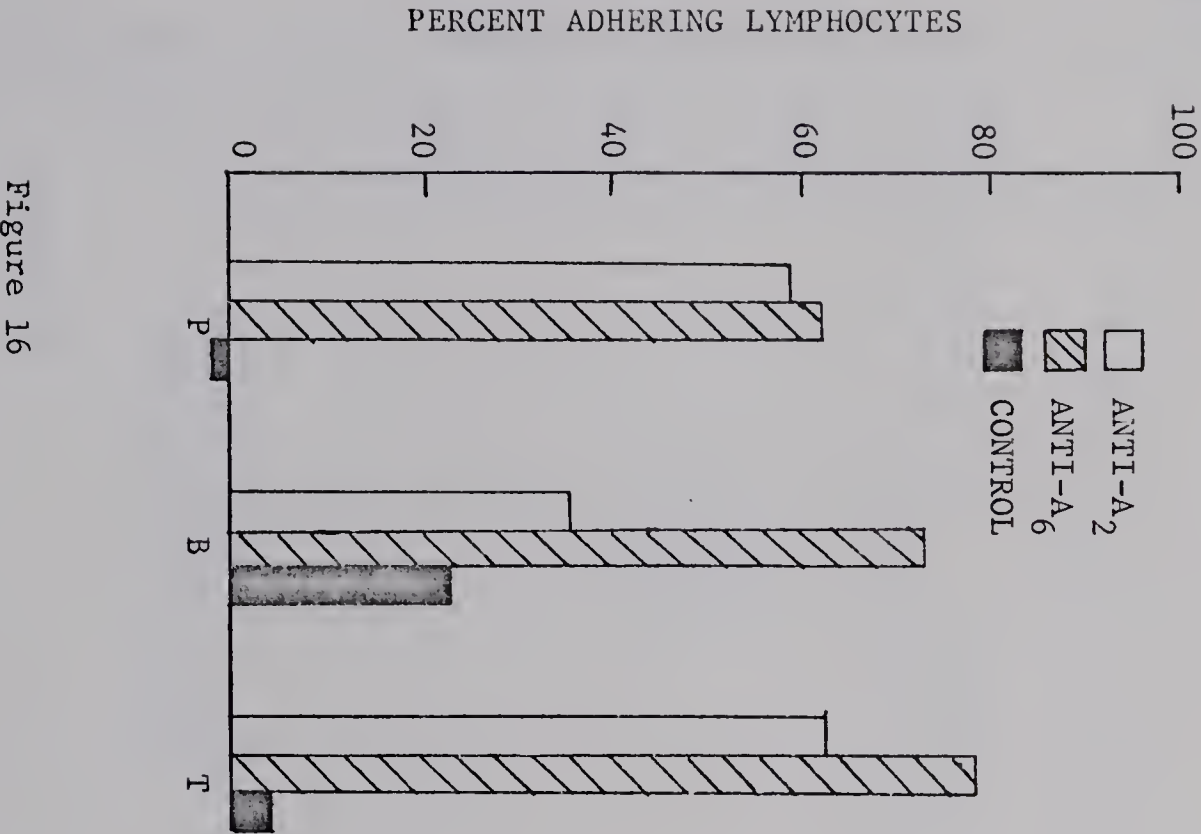


Figure 16

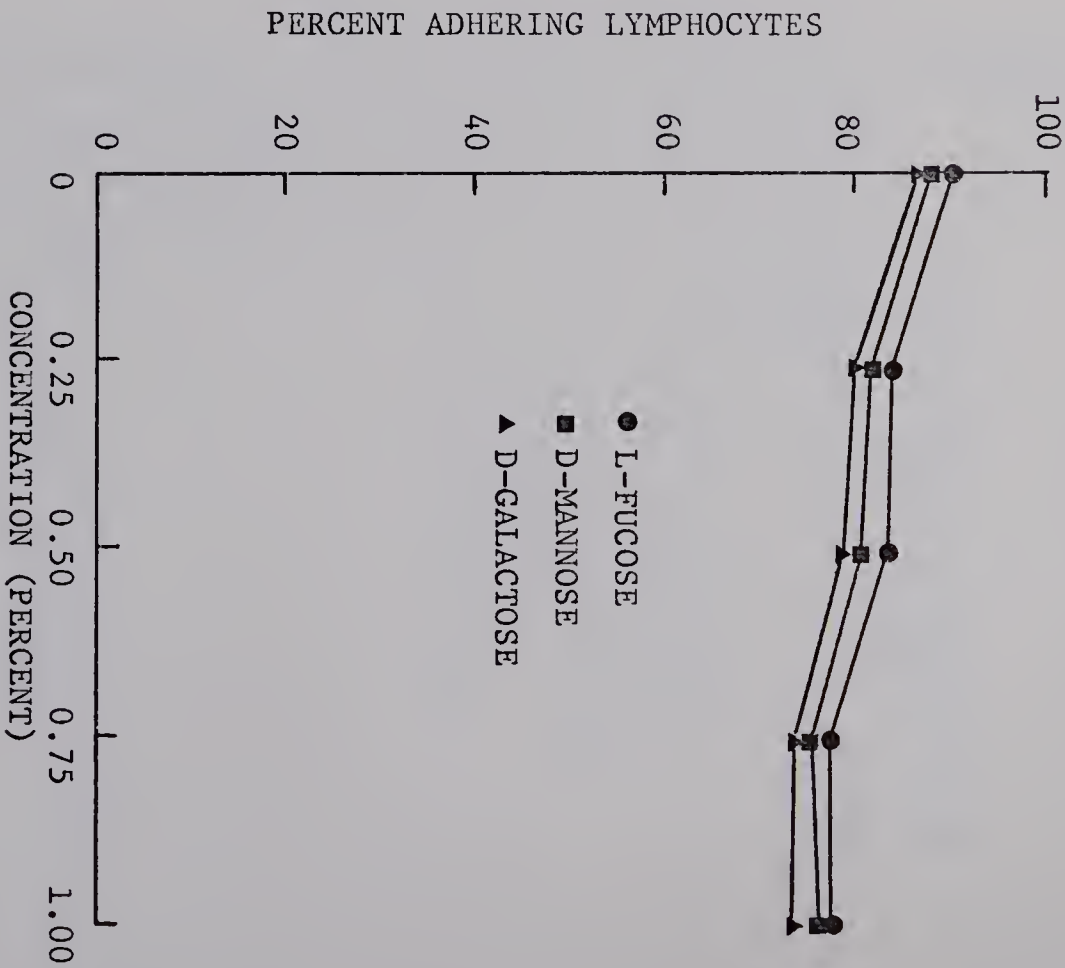


Figure 17







Figure 18. The effect of simple sugars on aspecific adherence.  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested in HBSS and the means of the 3 different tests are represented.

Figure 19. The effect of simple amines on allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$ . Three series of tests were performed on lymphocytes obtained from 3 different birds and each point represents the mean of the tests.

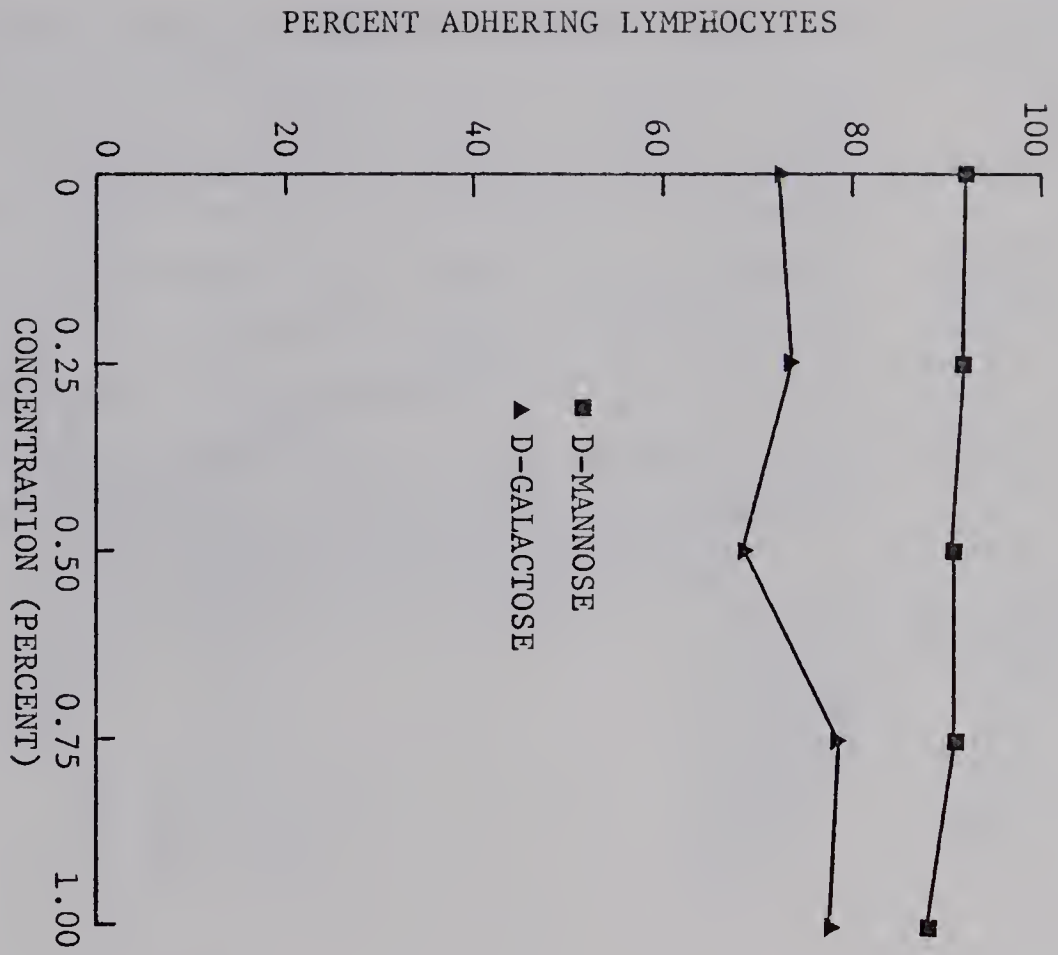


Figure 18

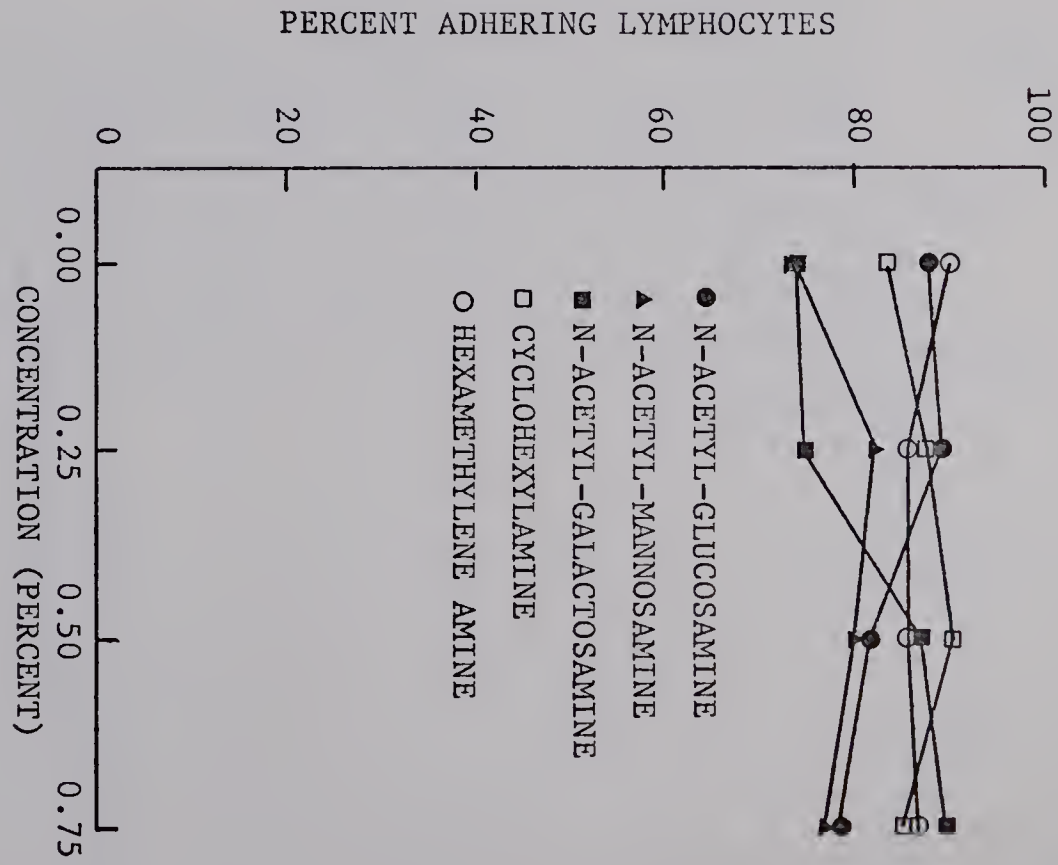


Figure 19







Figure 20. The effect of amino sugars on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$ . Three series of test were performed on lymphocytes obtained from 3 different birds and the means of the tests were represented. The pH was not adjusted for the tests represented with lines, but the pH was adjusted to 8.0 with 0.1N NaOH for the tests represented by bars indicating that the effect on allofixation must be attributed to the alteration of pH by the amines.

Figure 21. The effect of sodium borate on aspecific adherence (HBSS) and allofixation (AF).  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were used for the test of aspecific adherence, whereas  $\underline{B}^{13}/\underline{B}^{13}$  and  $\underline{B}^{13}/\underline{B}^{15}$  lymphocytes were tested with specific anti- $B_{13}$  serum. Three series of aspecific adherence tests and 4 series of allofixation tests were done, and the means of these tests were represented.

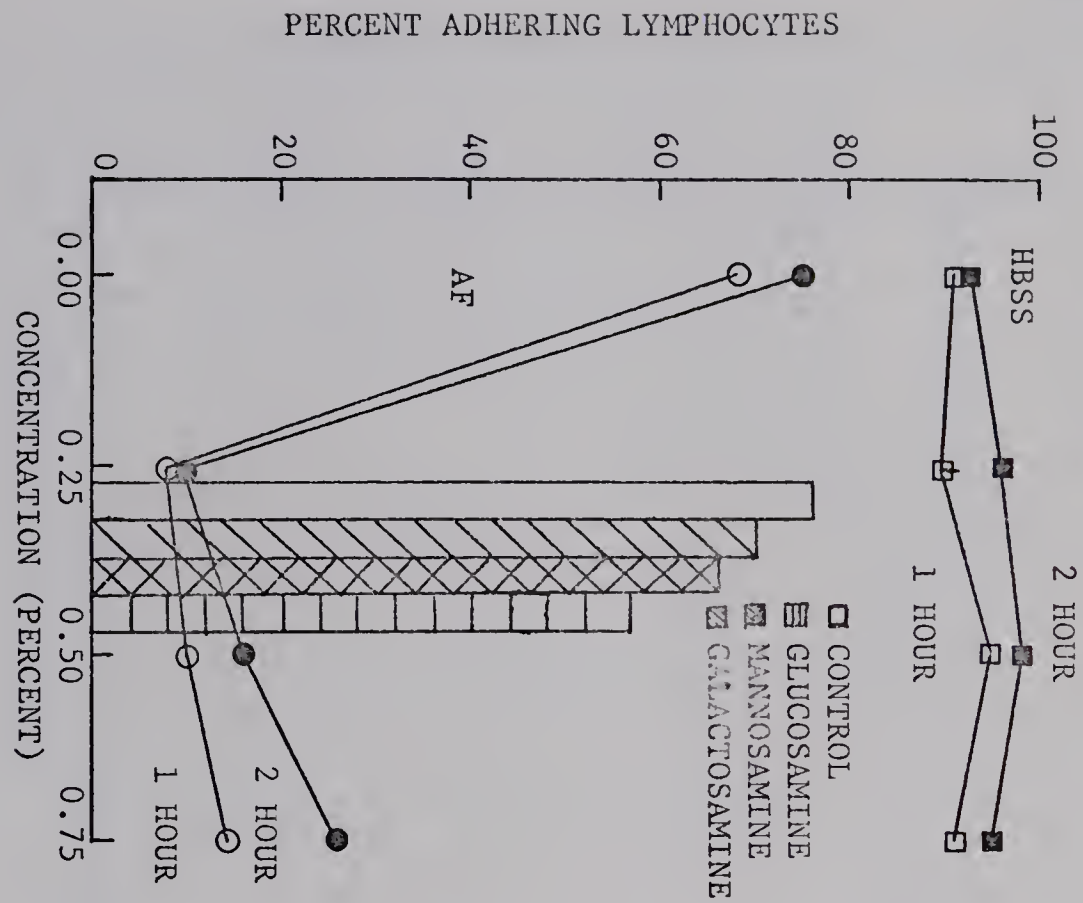


Figure 20

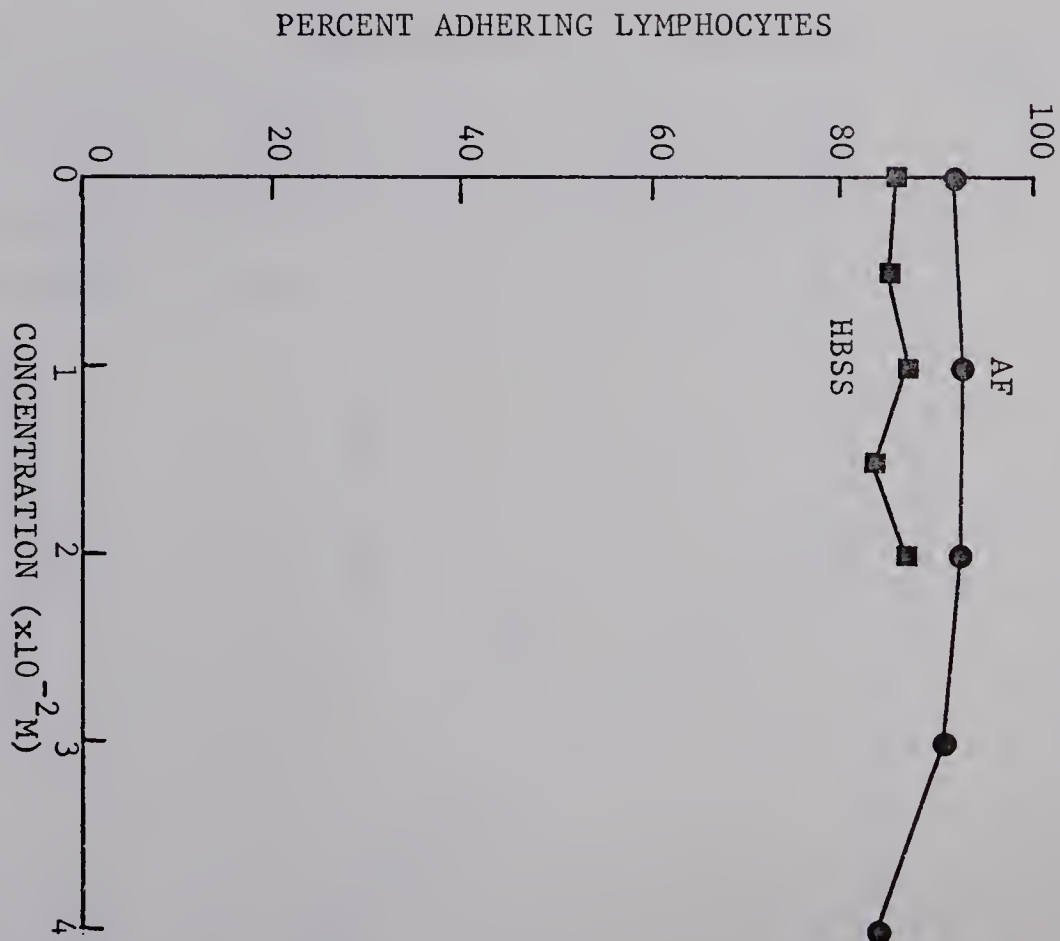


Figure 21





Figure 22. The effect of sodium periodate on aspecific adherence (HBSS) and allofixation (AF).  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were used for the test of aspecific adherence, whereas  $\underline{B}^{13}/\underline{B}^{13}$  and  $\underline{B}^{13}/\underline{B}^{15}$  lymphocytes were tested with specific anti- $B_{13}$  serum. Three series of aspecific adherent tests and 4 series of allofixation tests were done, and the means of these tests were represented.

Figure 23. The effect of hydrocortisone on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used in allofixation test. Three series of tests were done for aspecific adherence test and allofixation test. The means of these tests were taken.



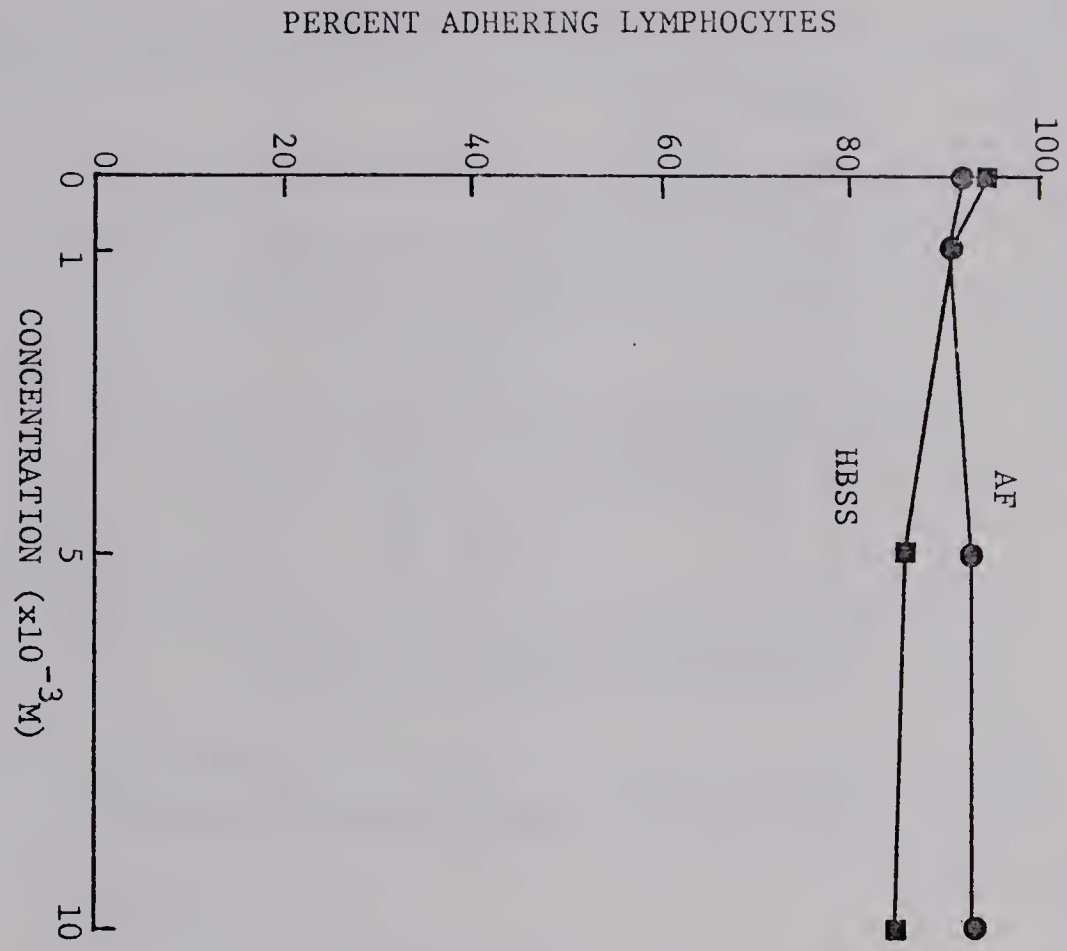


Figure 22

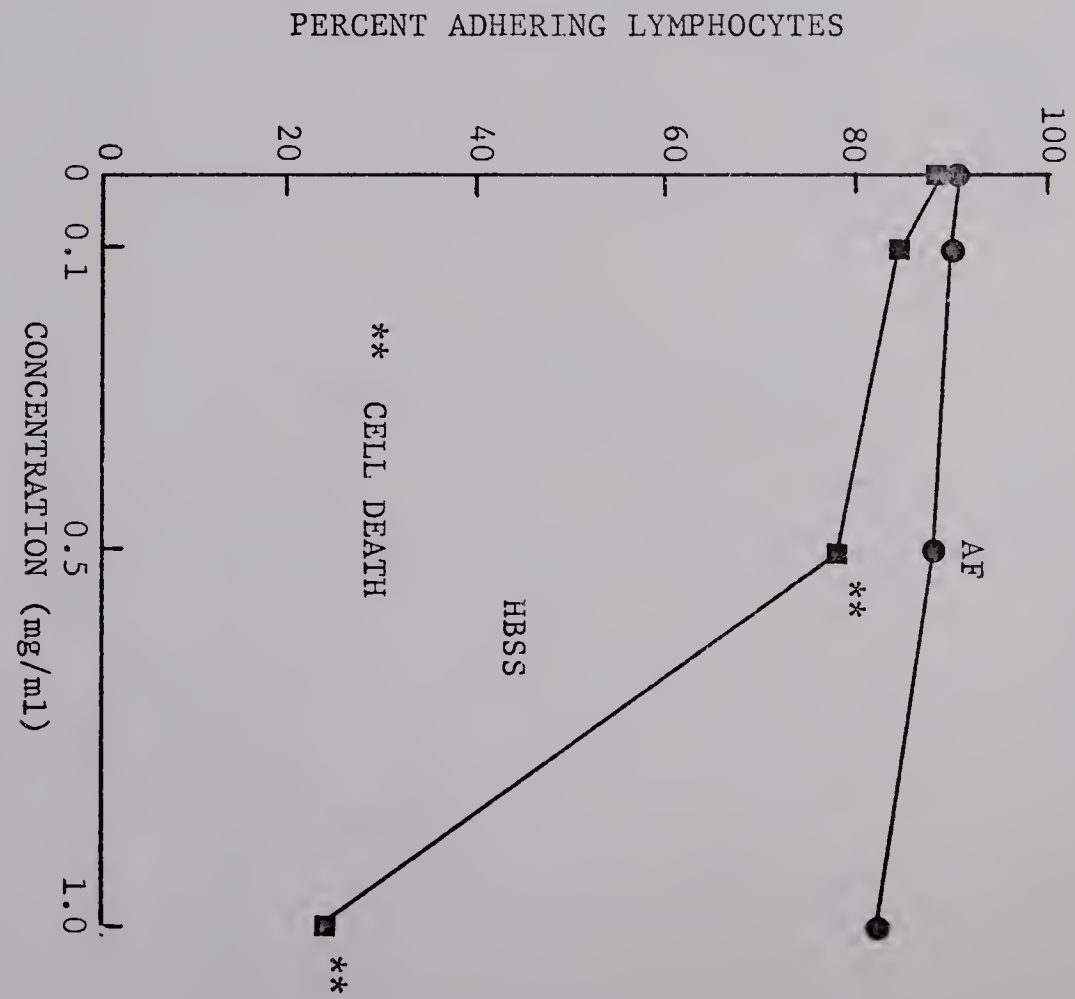


Figure 23





Figure 24. The allofixation of trypsinized lymphocytes with respect to change of pH.

- (a)  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested with specific anti- $B_{14}$  serum at room temperature for 2 hours.
- (b)  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes were tested with specific anti- $B_{15}$  serum at room temperature for 2 hours.
- (c)  $\underline{B}^2/\underline{B}^2$  lymphocytes were tested with specific anti- $B_2$  serum at room temperature for 2 hours.

Three series of tests were performed in each case, and the means of the 3 tests were presented.

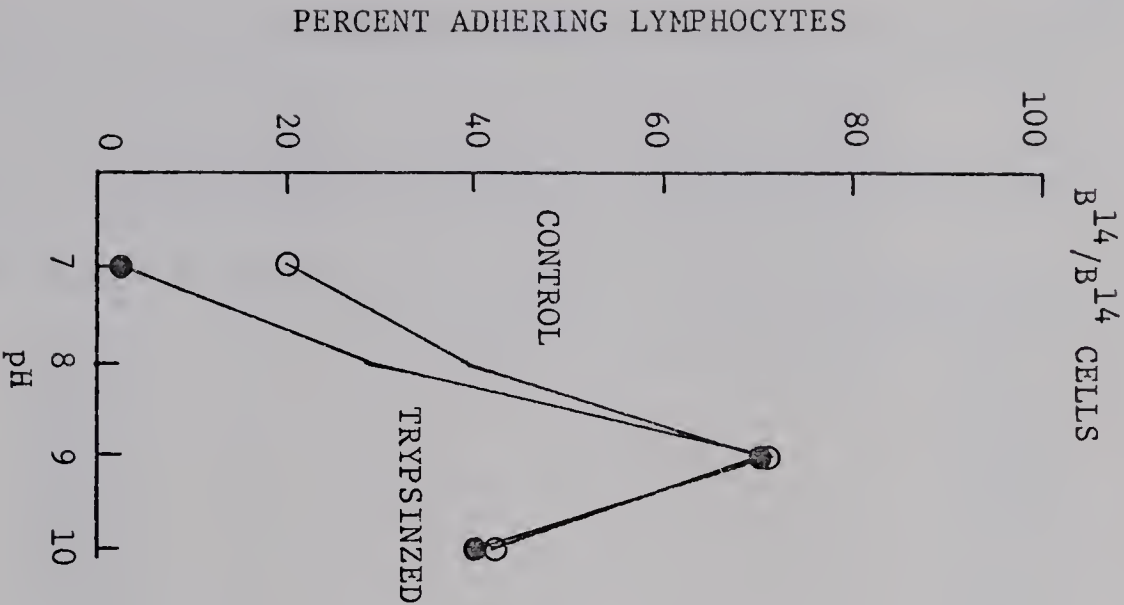


Figure 24a

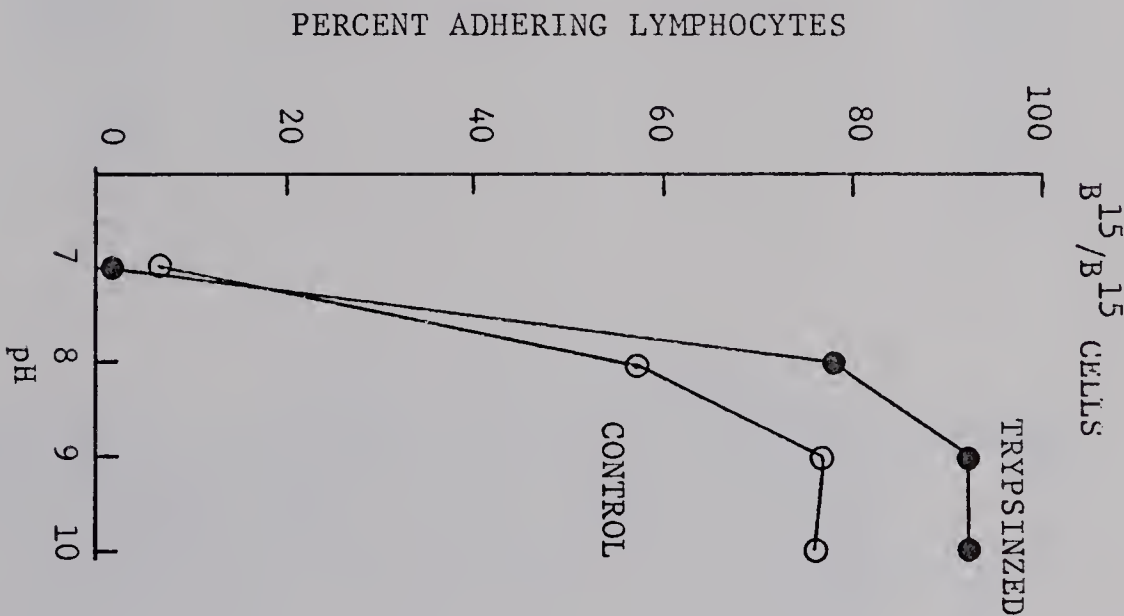


Figure 24b

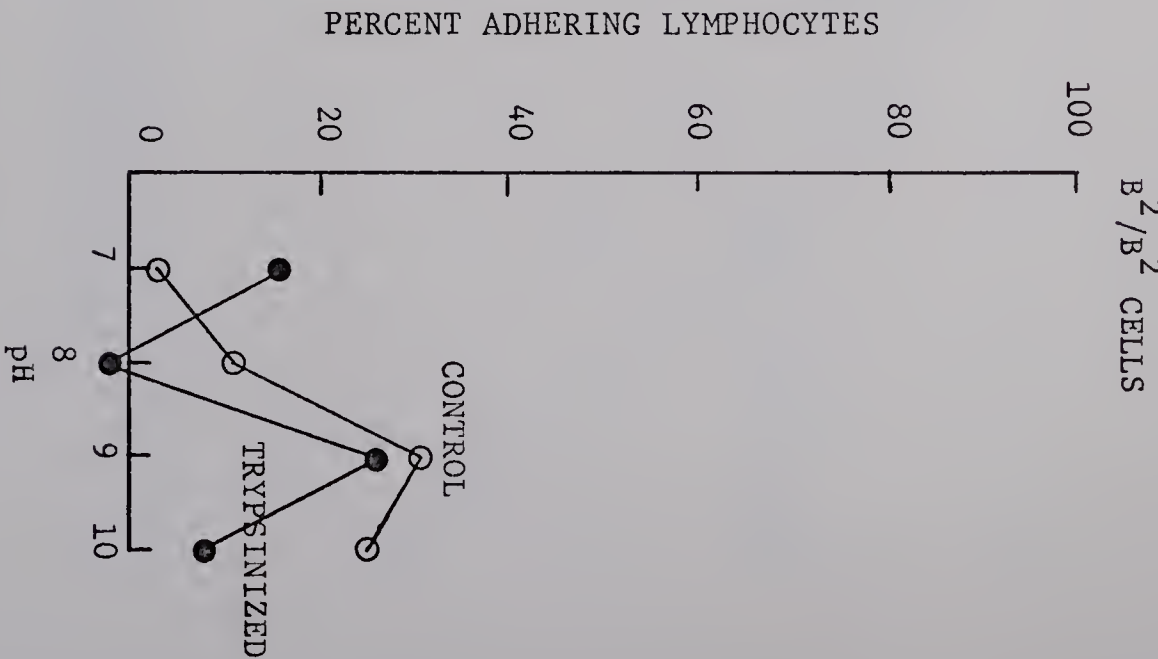


Figure 24c







Figure 25. The effect of metabolic inhibitor, 2-deoxy-D-glucose, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used in allofixation test. Four series of aspecific adherence and allofixation tests were performed. The means of these tests were taken.

Figure 26. The effect of metabolic inhibitor, iodoacetamide, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used in allofixation test. Two series of aspecific adherence and allofixation tests were done and the means of the tests were taken.

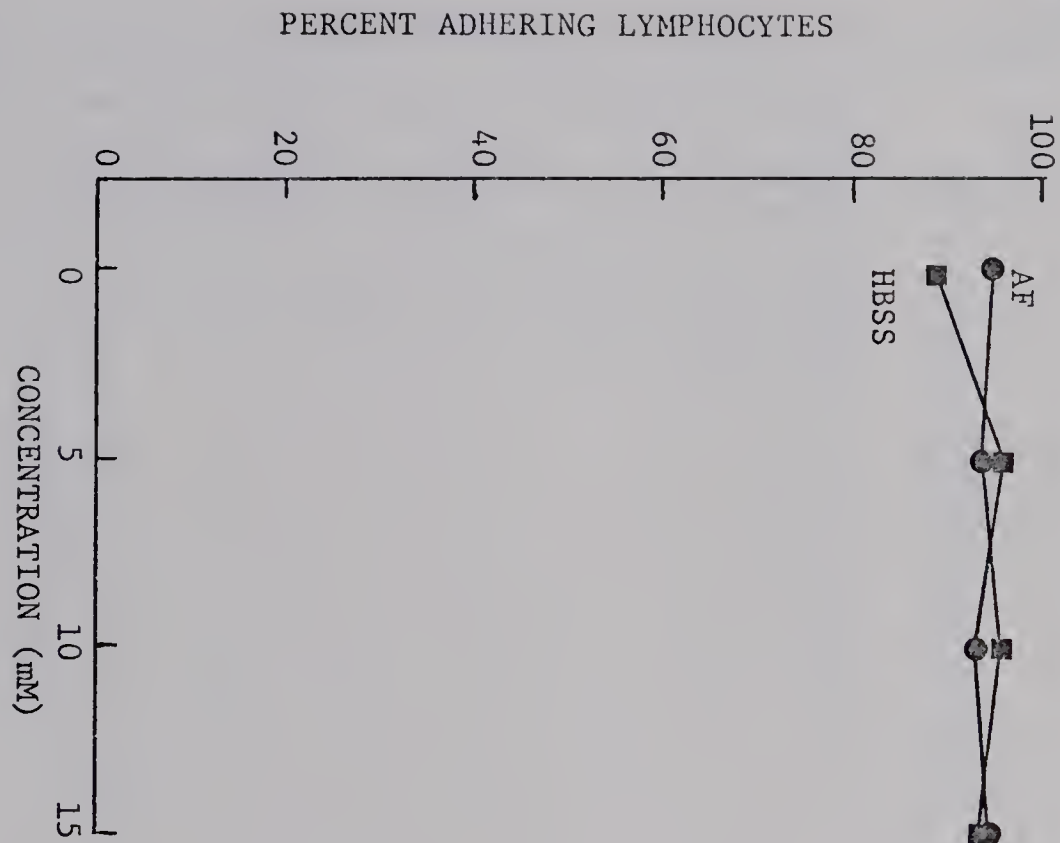


Figure 25

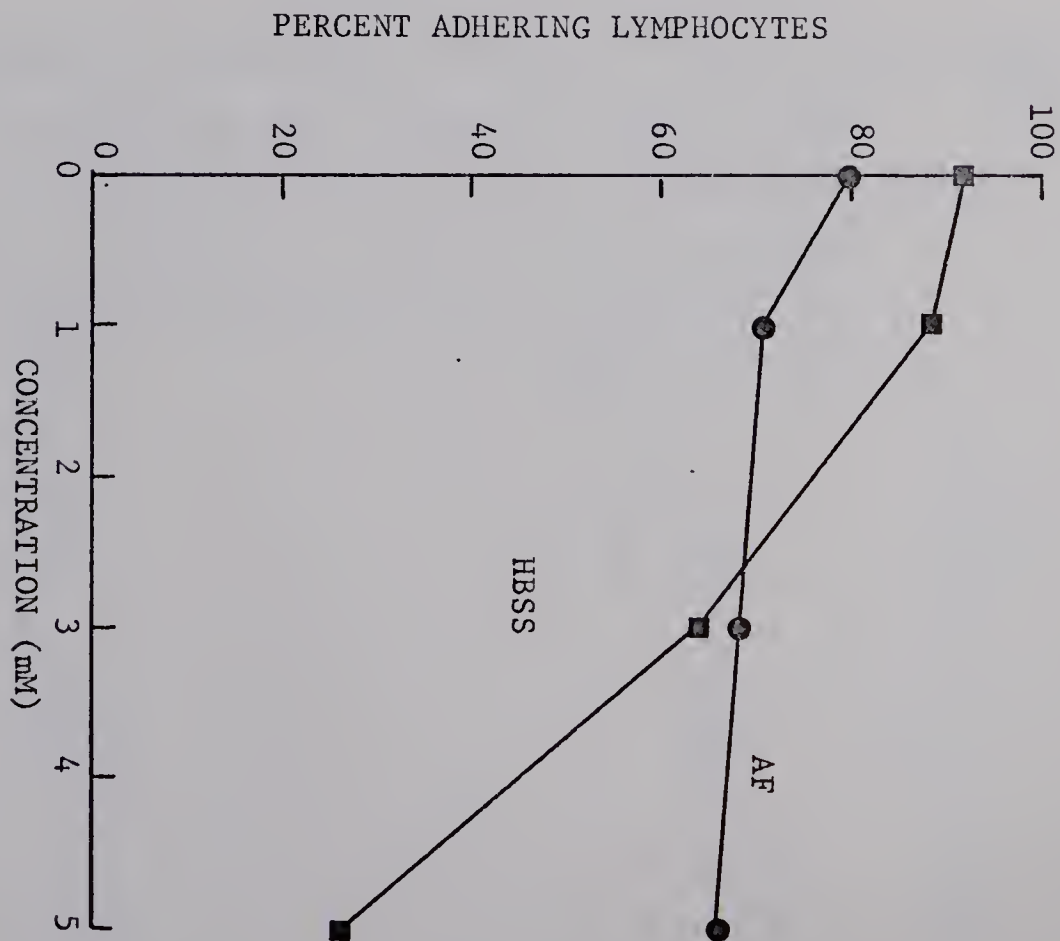


Figure 26





Figure 27. The effect of metabolic inhibitor, sodium cyanide, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used for allofixation test. Two series of aspecific adherence tests and allofixation tests were done and the means of the tests were taken.

Figure 28. The effect of metabolic inhibitor, 2,4 dinitrophenol, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used for allofixation test. Two series of aspecific adherence and allofixation tests were performed and the means of these tests were taken.



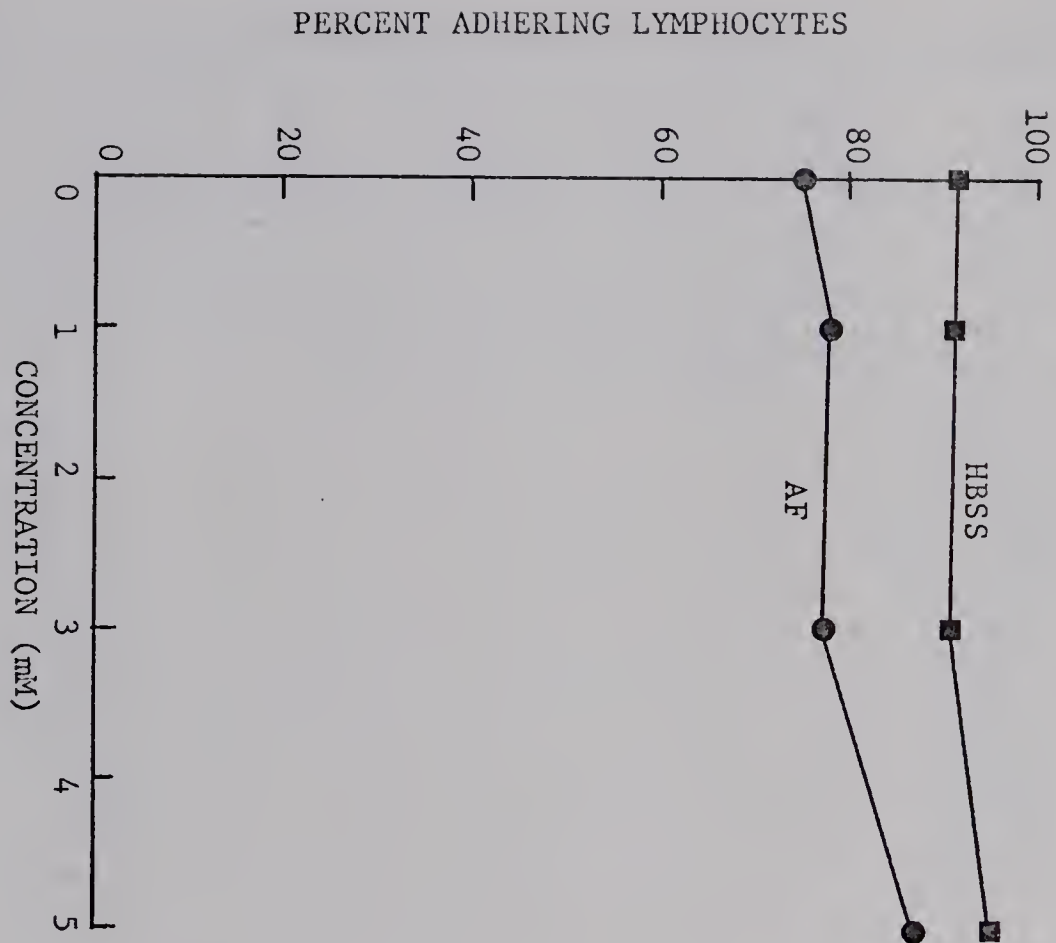


Figure 27

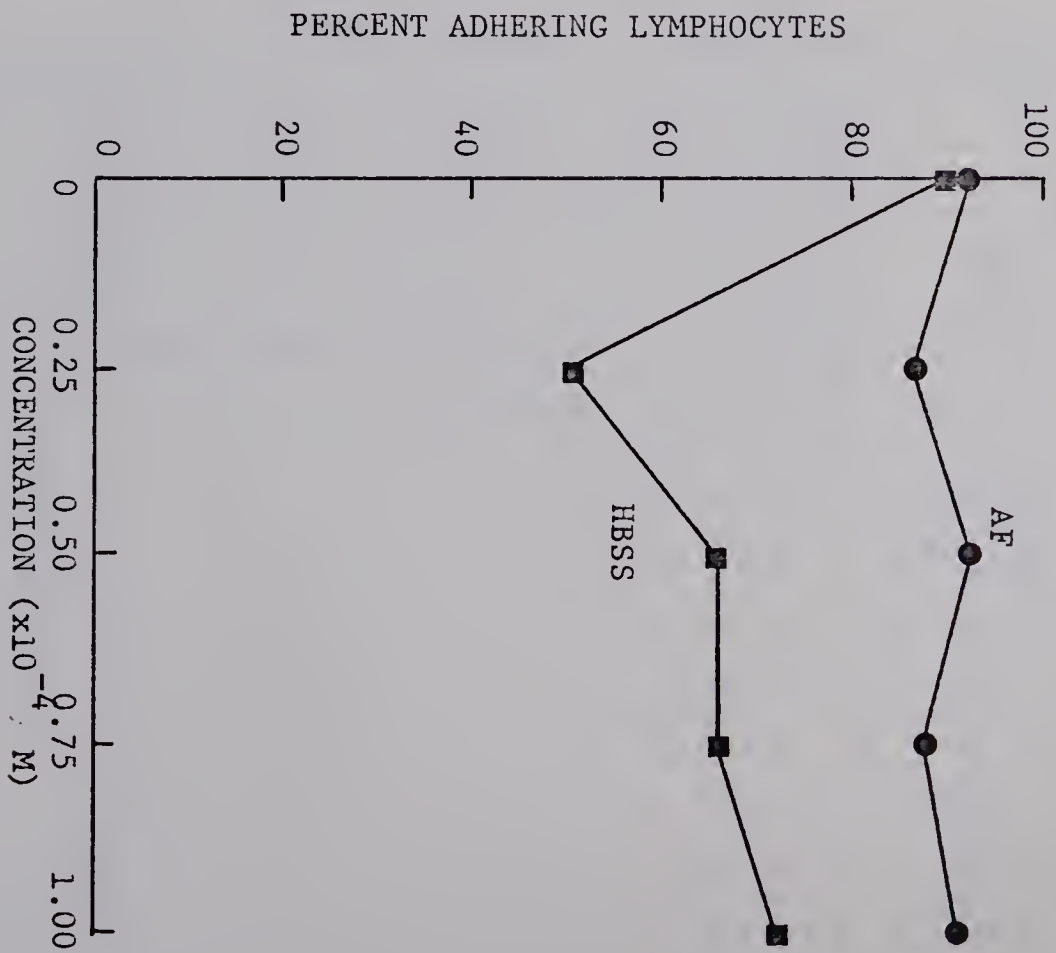


Figure 28





Figure 29. The effect of vinblastine, an inhibitor of microtubules, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Specific anti- $B_{14}$  serum was used for allofixation test. Three series of allofixation and 1 series of aspecific adherence tests were performed and the means of the former tests were taken.

Figure 30. The effect of X-irradiation on allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Lymphocytes exposed to 500R (25R/min for 20 minutes), 1000R (200R/min for 5 minutes) and 5000R (200R/min for 25 minutes) were tested with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  bird. Duplicate tests were performed with the untreated and X-irradiated lymphocytes at room temperature for 2 hours and at pH 8.5.

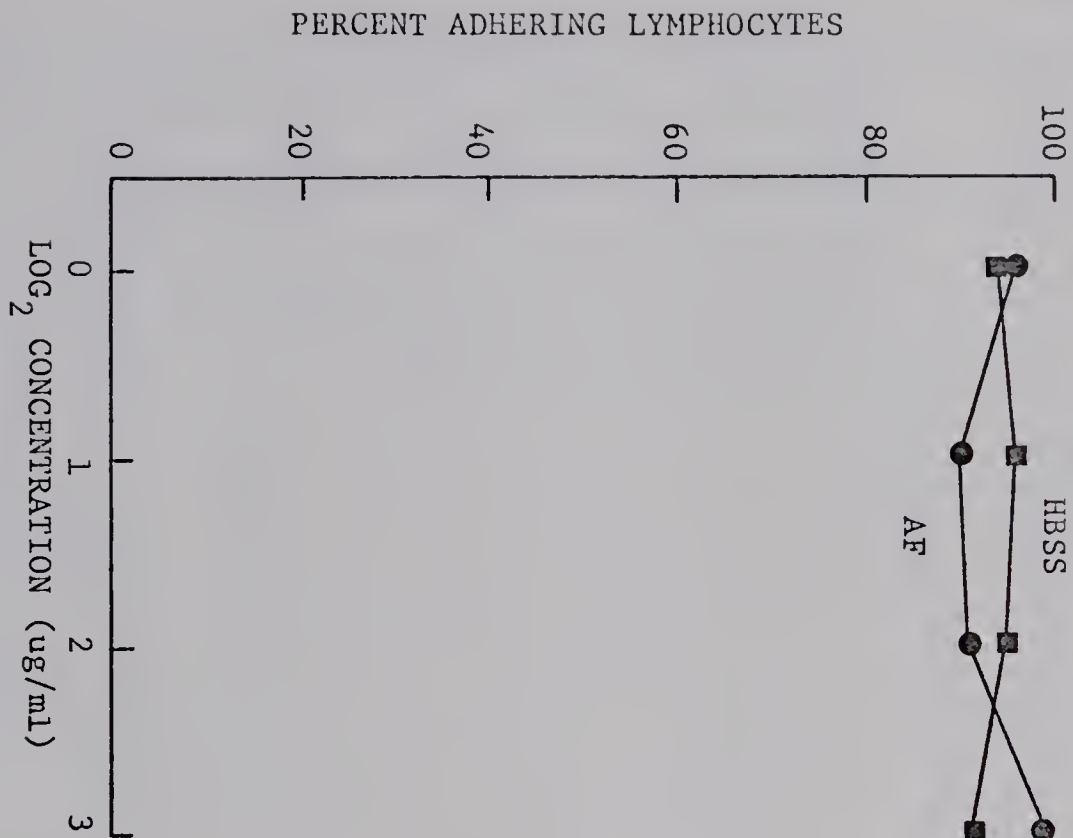


Figure 29

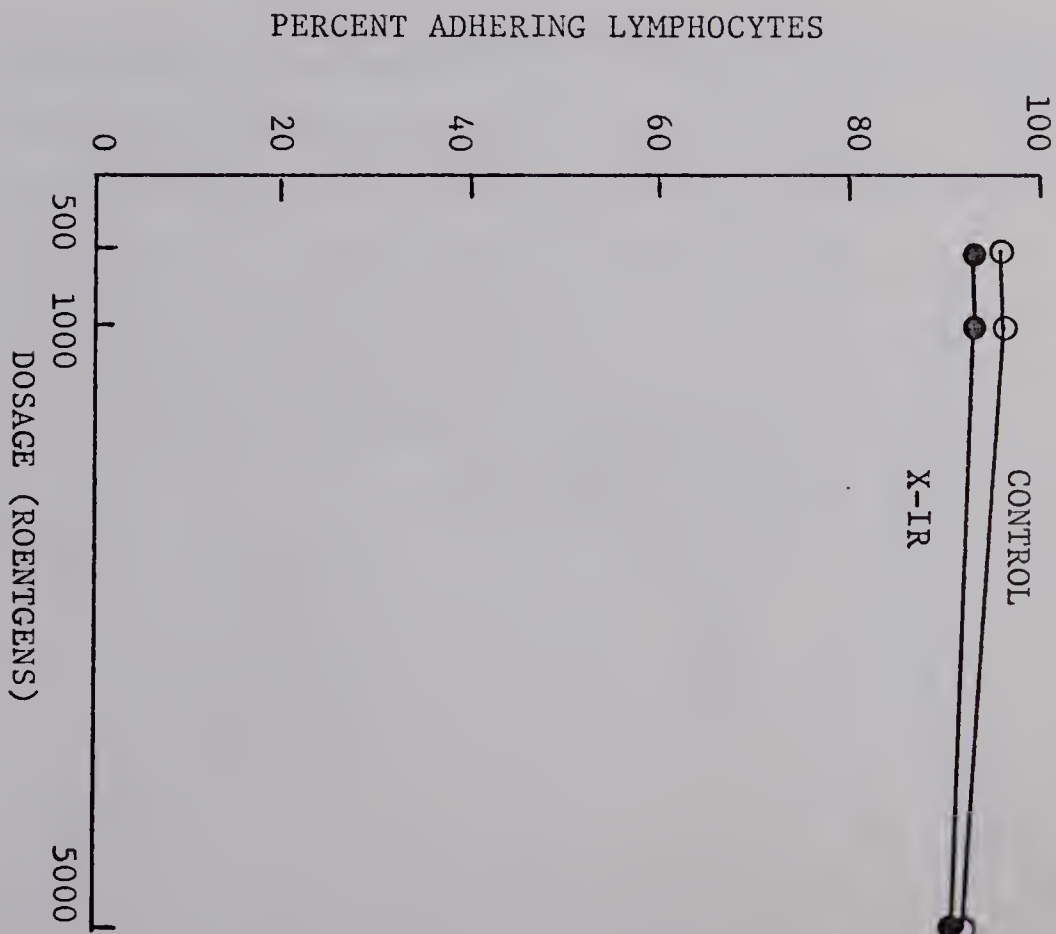


Figure 30







Figure 31. The effect of chloramphenicol, a protein inhibitor, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used for allofixation test. Three series of aspecific adherence and allofixation tests were performed and the means of these tests were taken.

Figure 32. The effect of anticoagulants on allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^{13}/\underline{B}^{13}$  was used for the tests. Two series of allofixation tests were performed and the means of the tests were taken. sodium citrate, EDTA were tested at 10, 20 and 30 mM and heparin at 5, 25 and 50 units (USP)/ml. Sucrose was used as the control to eliminate the effect of increase in molarity of the medium.

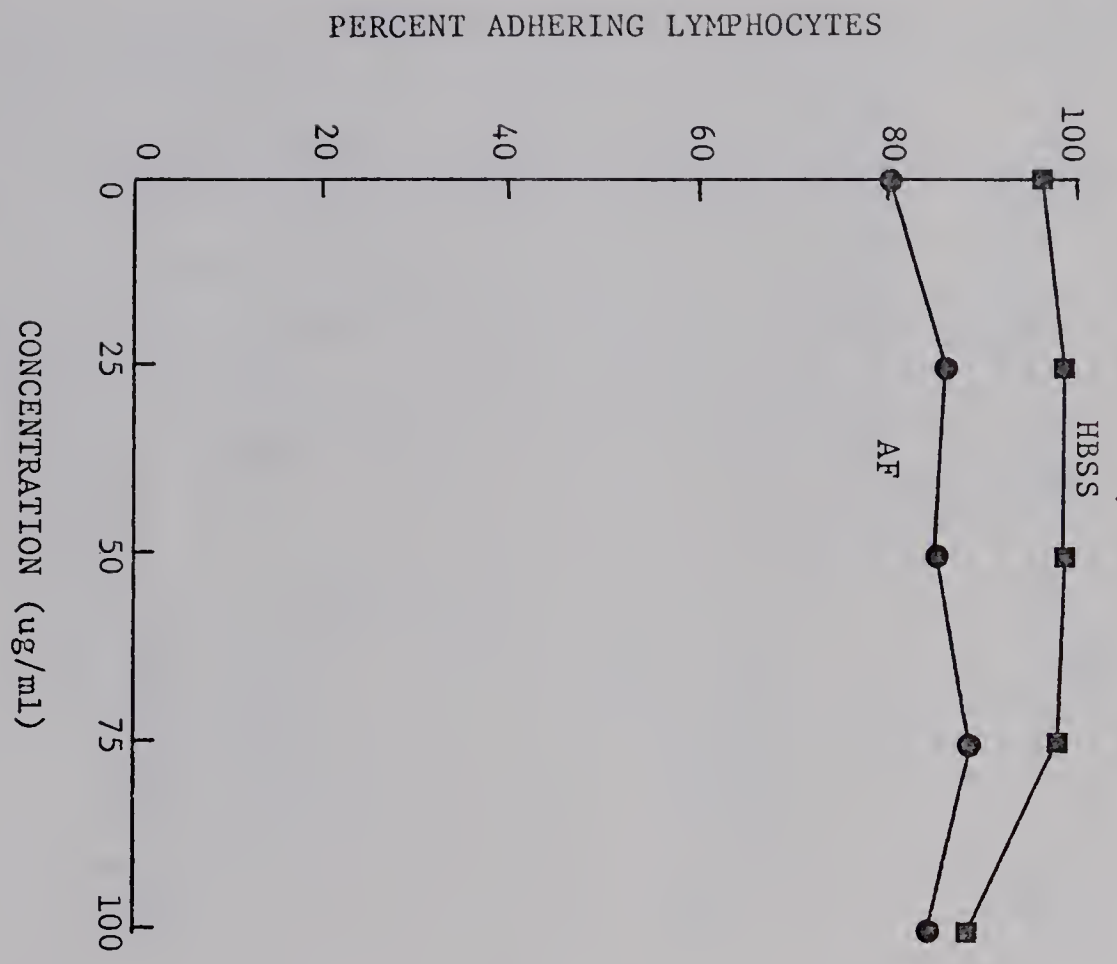


Figure 31

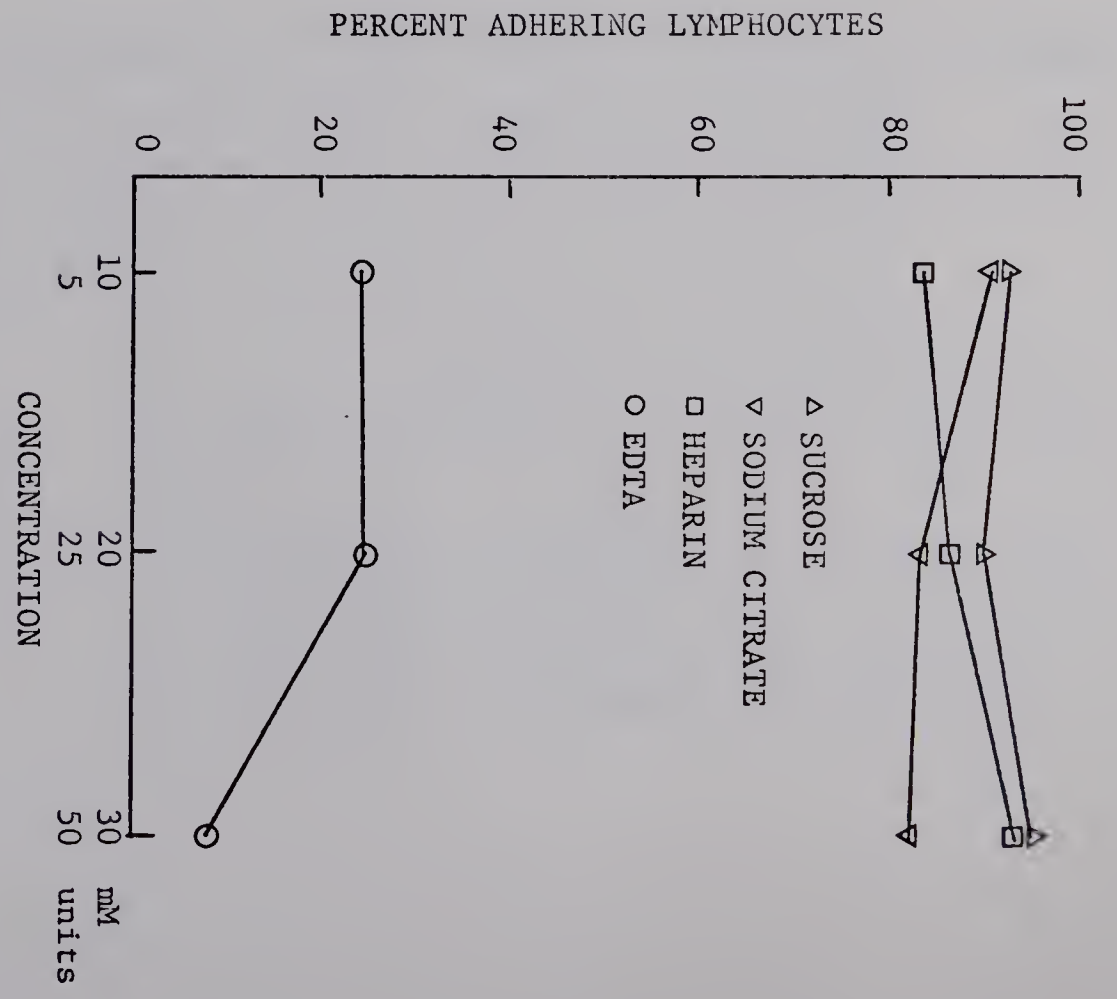


Figure 32





Figure 33. The effect of chelating agent, EDTA, on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $\underline{B}_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  was used for allofixation test. Duplicate tests were done in each case with the pH adjusted to 7.0 for the aspecific adherence test and to 8.0 for the allofixation test. The tests were performed at room temperature for 2 hours, and viable cells were observed and counted under phase contrast microscope.

Figure 34. The effect of metallic ions on the inhibition of allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes induced by EDTA. Unabsorbed anti- $\underline{B}_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  was tested in the presence of 5 mM EDTA, 5 mM EDTA plus 5 mM metallic ions or without EDTA. Duplicate tests were done at room temperature for 2 hours and the means of the tests were taken.



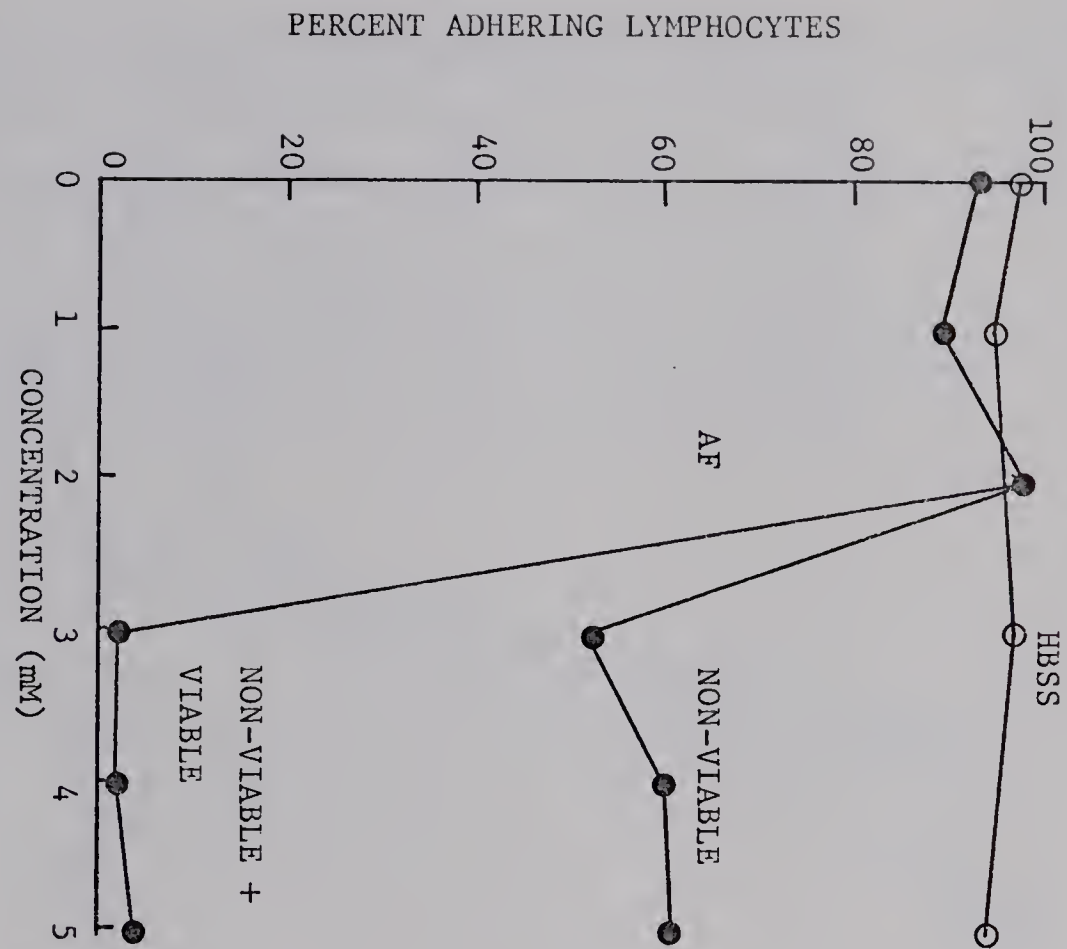


Figure 33

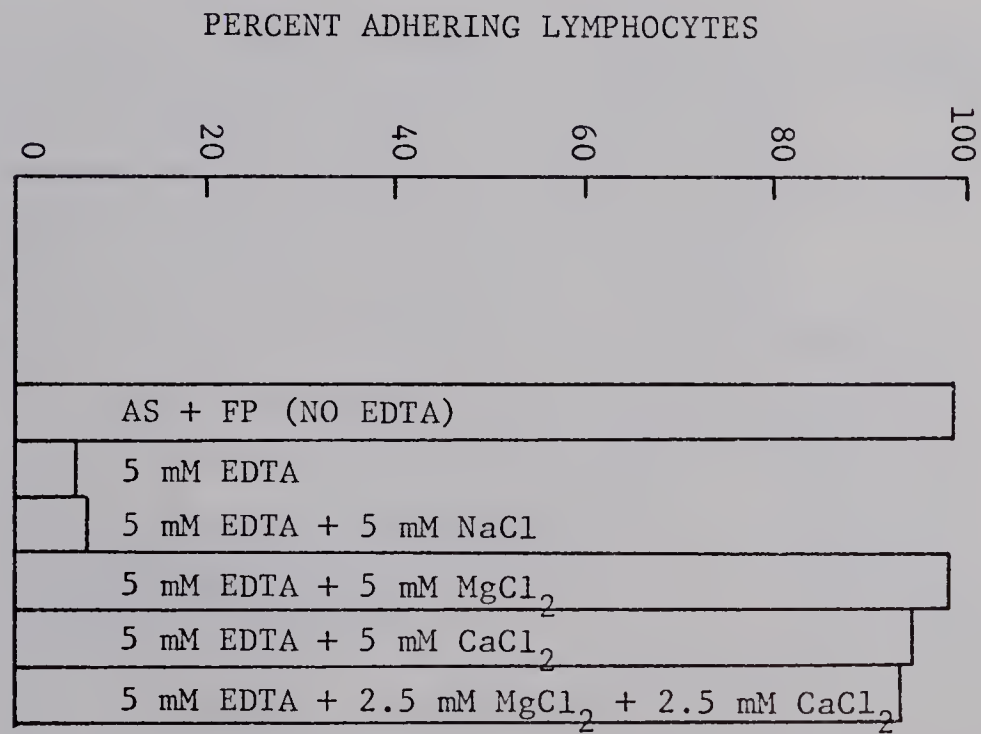


Figure 34





Figure 35. The effect of concentration of divalent cations on the inhibition of allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes in the presence of 5 mM EDTA. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  was tested in the presence of 5 mM EDTA, 5 mM EDTA plus 1, 2, 3 and 4 mM divalent cations at room temperature for a period of 2 hours. Two series of the test were performed and the means of the tests were taken,

Figure 36. The effect of plastic or glass substrates on allofixation of  $\underline{B}^{14}/\underline{B}^{14}$  and  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes. One unabsorbed anti- $B_{14}$ , one specific anti- $B_{14}$  and two specific anti- $B_{15}$  sera were employed in the test. Duplicate or triplicate tests were performed with each of the 4 sera at room temperature for 2 hours and at pH 8.5. A total of 17 tests were done and the means of these tests were used.

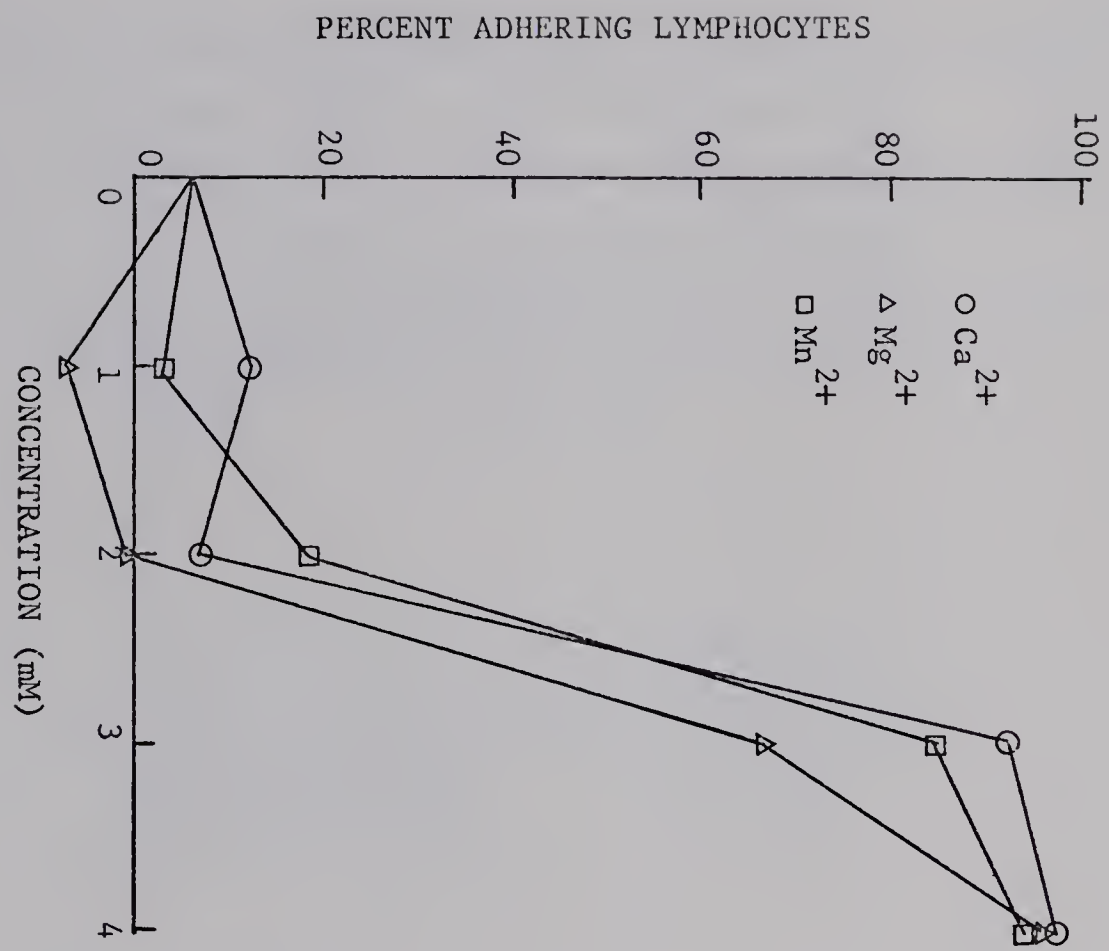


Figure 35

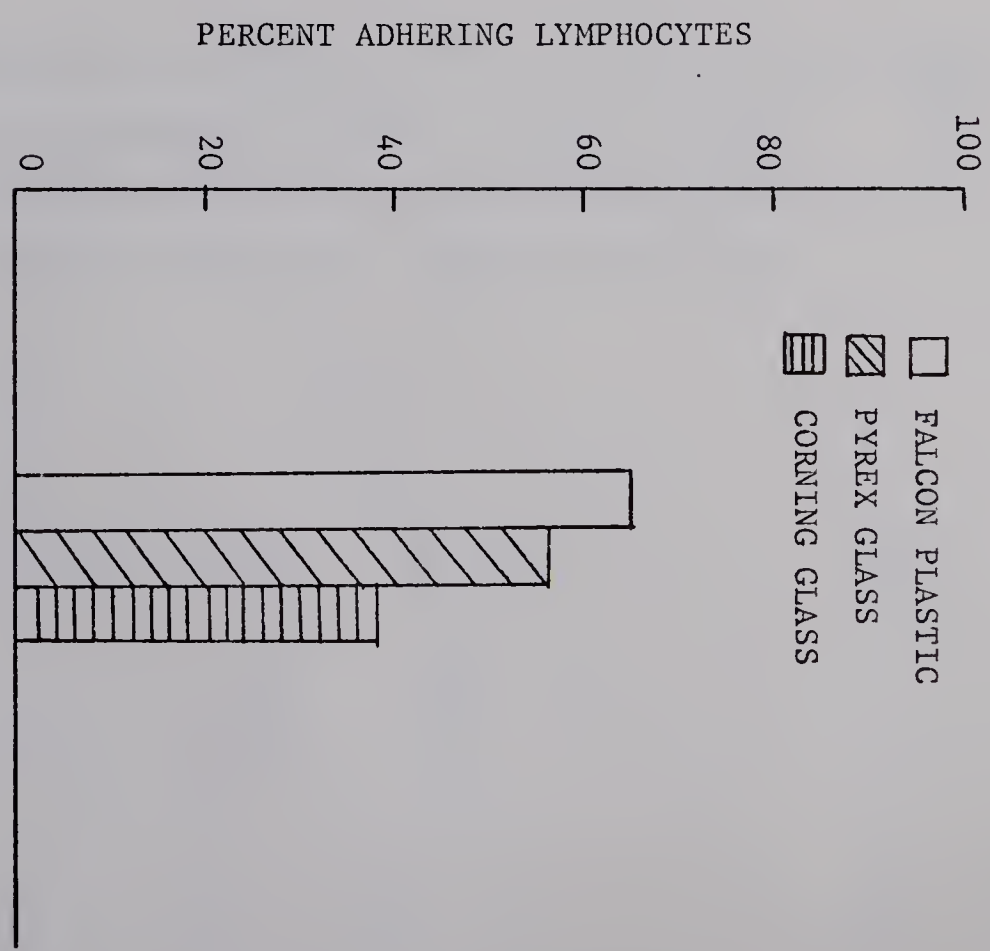


Figure 36







Figure 37. The effect of coated plastic on aspecific adherence (HBSS) and allofixation (AF) of  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes. Specific anti- $B_{15}$  serum was used for the allofixation test. Plastic tubes were coated with 0.5% gelatin, BSA or agar or untreated. Three or more tests were performed on each type of the coated surfaces and the means of the tests were used.

Figure 38. The effect of pH on the aspecific adherence to coated plastic surfaces.  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes were used for the test and two or three series of the aspecific adherence test were performed. The means of these tests were used.

PERCENT ADHERING LYMPHOCYTES

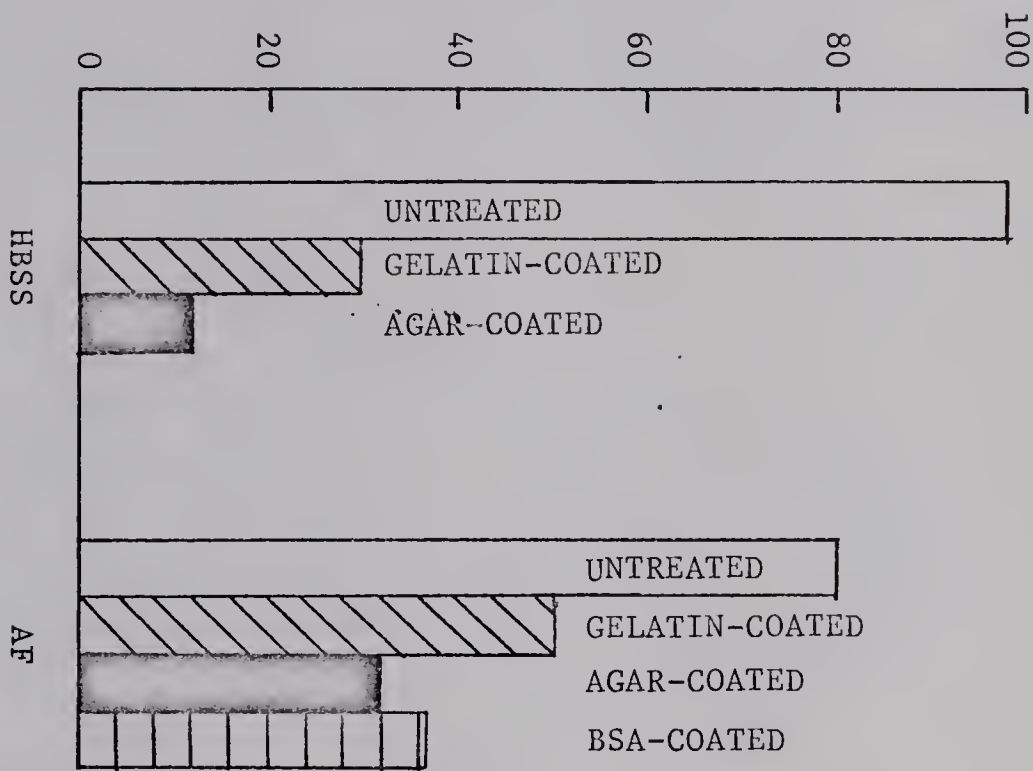


Figure 37

PERCENT ADHERING LYMPHOCYTES

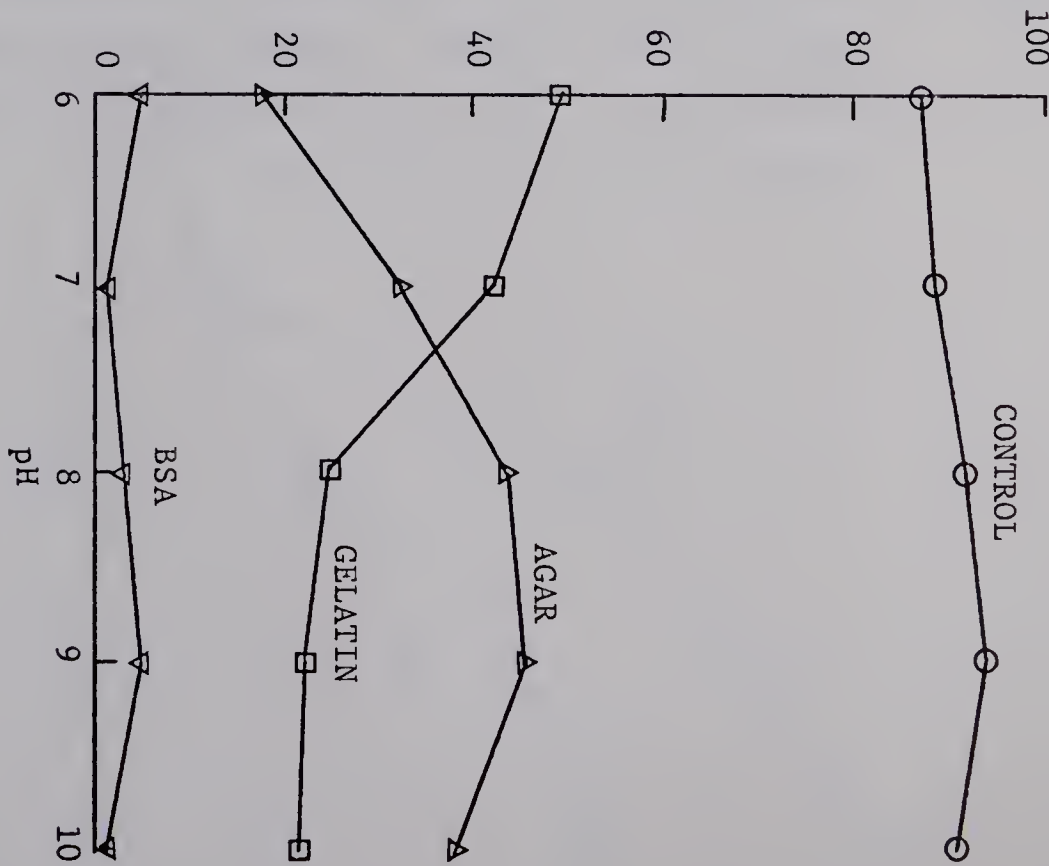


Figure 38





Figure 39. The effect of pH on the allofixation of  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes to gelatin-coated plastic. Specific anti- $B_{15}$  serum was used for the test. Three series of allofixation tests were performed and the means of these tests were used.

Figure 40. The effect of pH on allofixation of  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes to BSA-coated plastic. Specific anti- $B_{15}$  serum was used for the test. Two series of allofixation tests were done and the means of these tests were used.



PERCENT ADHERING LYMPHOCYTES

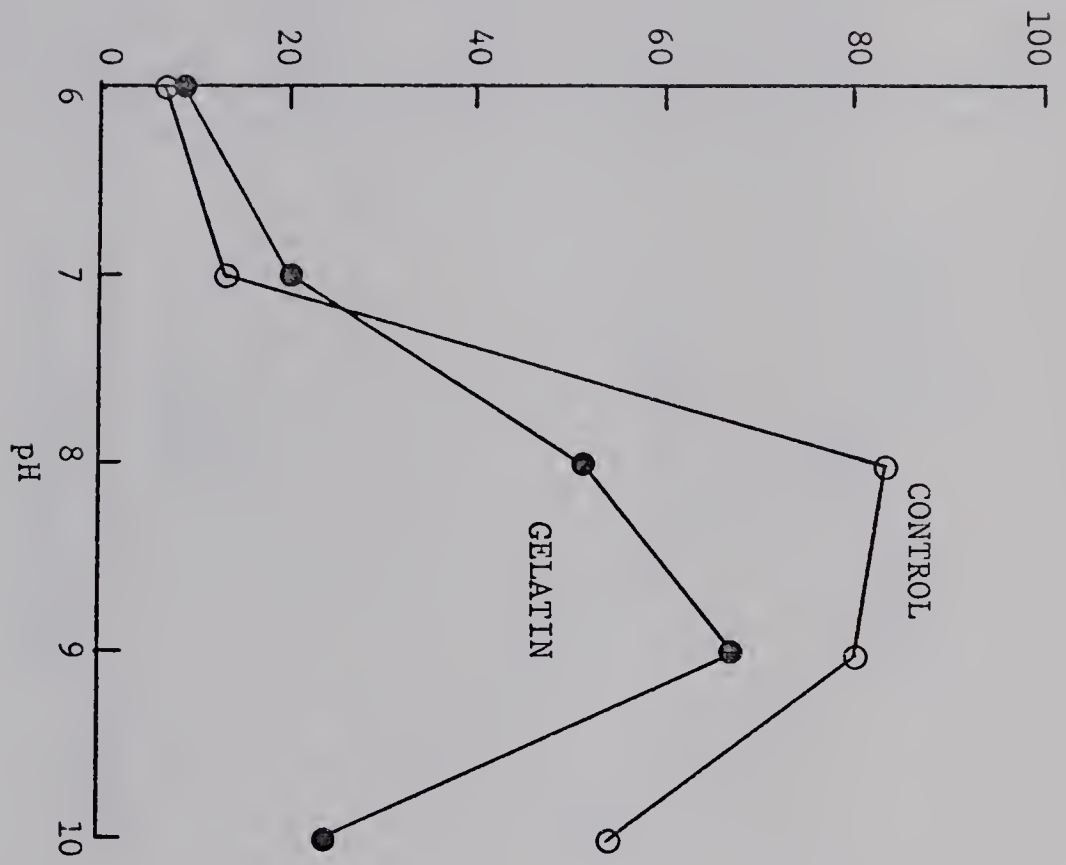


Figure 39

PERCENT ADHERING LYMPHOCYTES

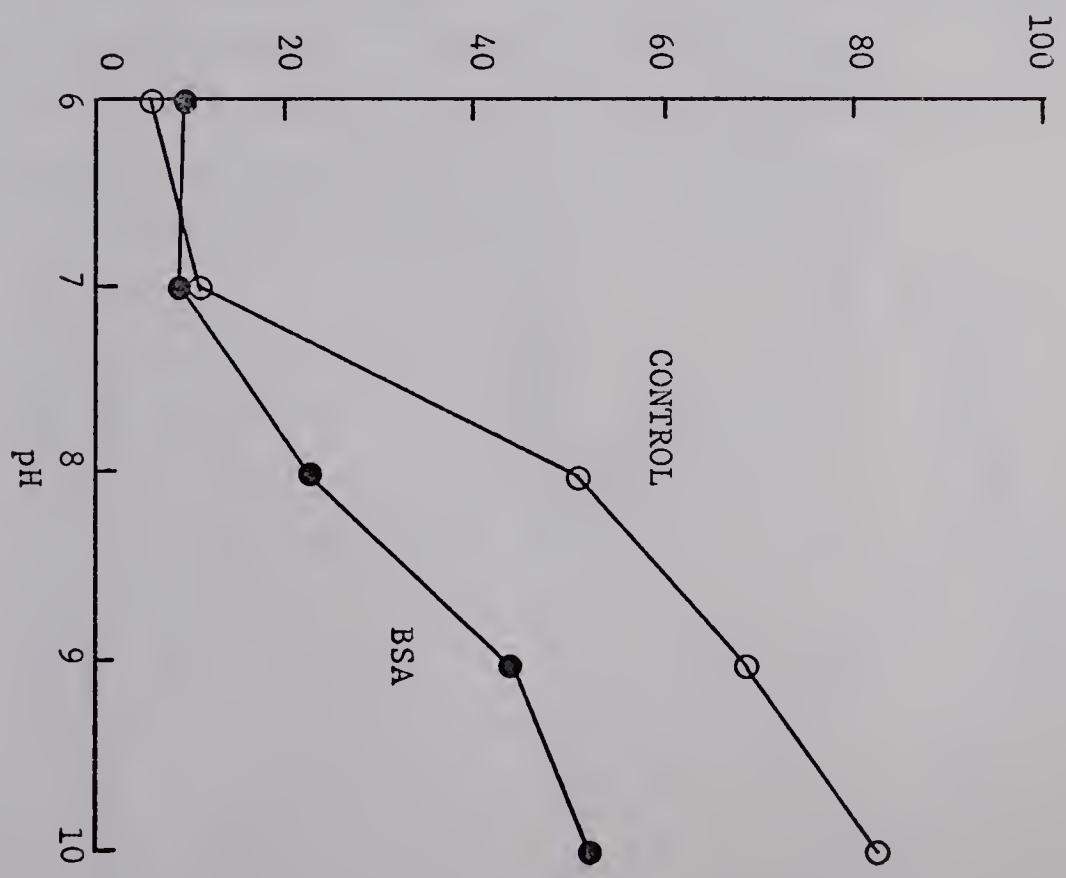


Figure 40





Figure 41a. Allofixation by primary, secondary and tertiary antisera on  $\underline{B}^2/\underline{B}^2$  lymphocytes. Unabsorbed anti- $B_2$  prepared in  $\underline{B}^1/\underline{B}^{14}$  was used for the tests. The tests were done at room temperature for 2 hours and at pH 8.5.

Figure 41b. Allofixation by primary, secondary and tertiary antisera on  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes. Unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^1/\underline{B}^2$  was used for the tests. Five series of tests were performed at room temperature for 2 hours and at pH 8.5. The means of these tests were used.

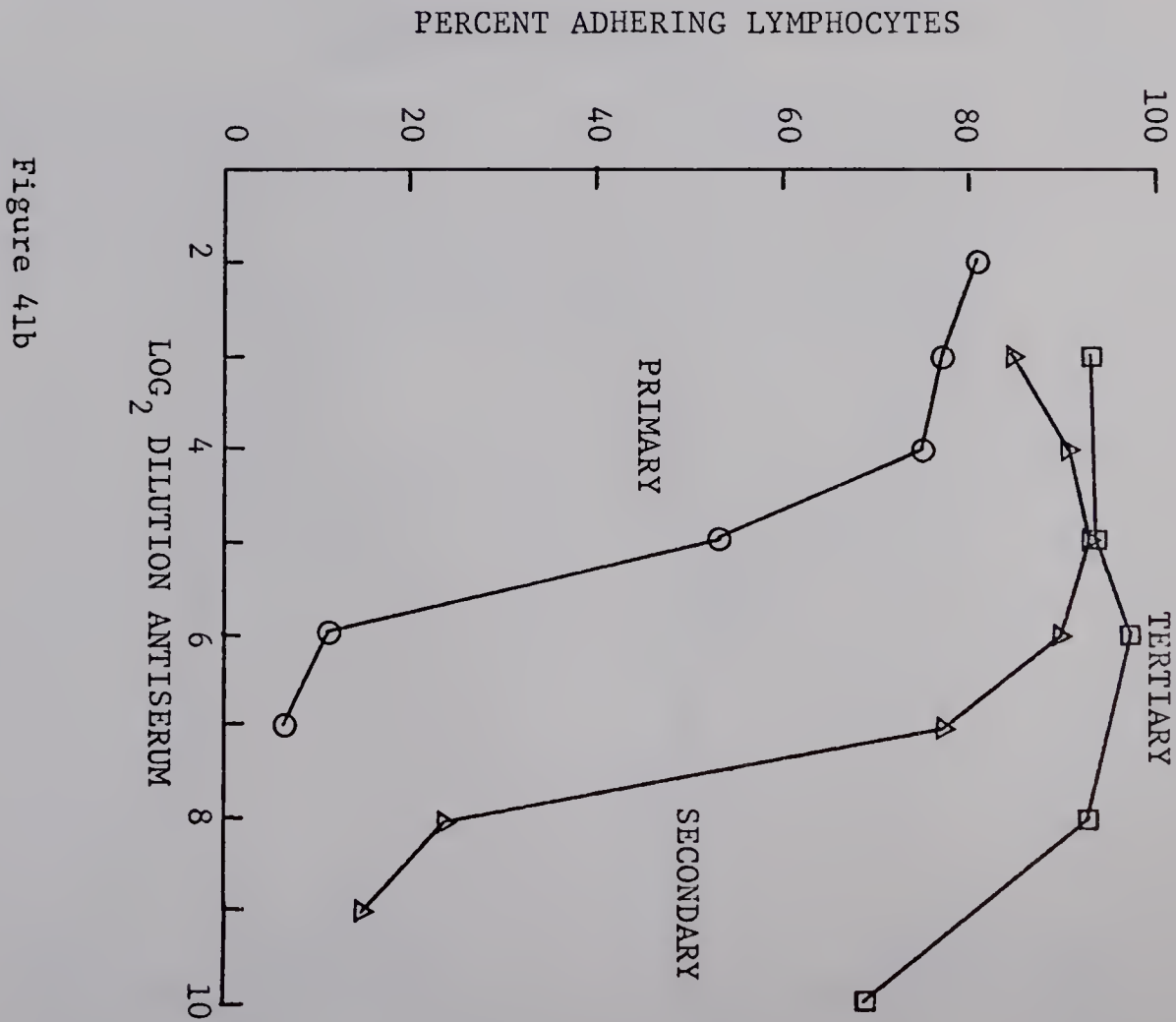
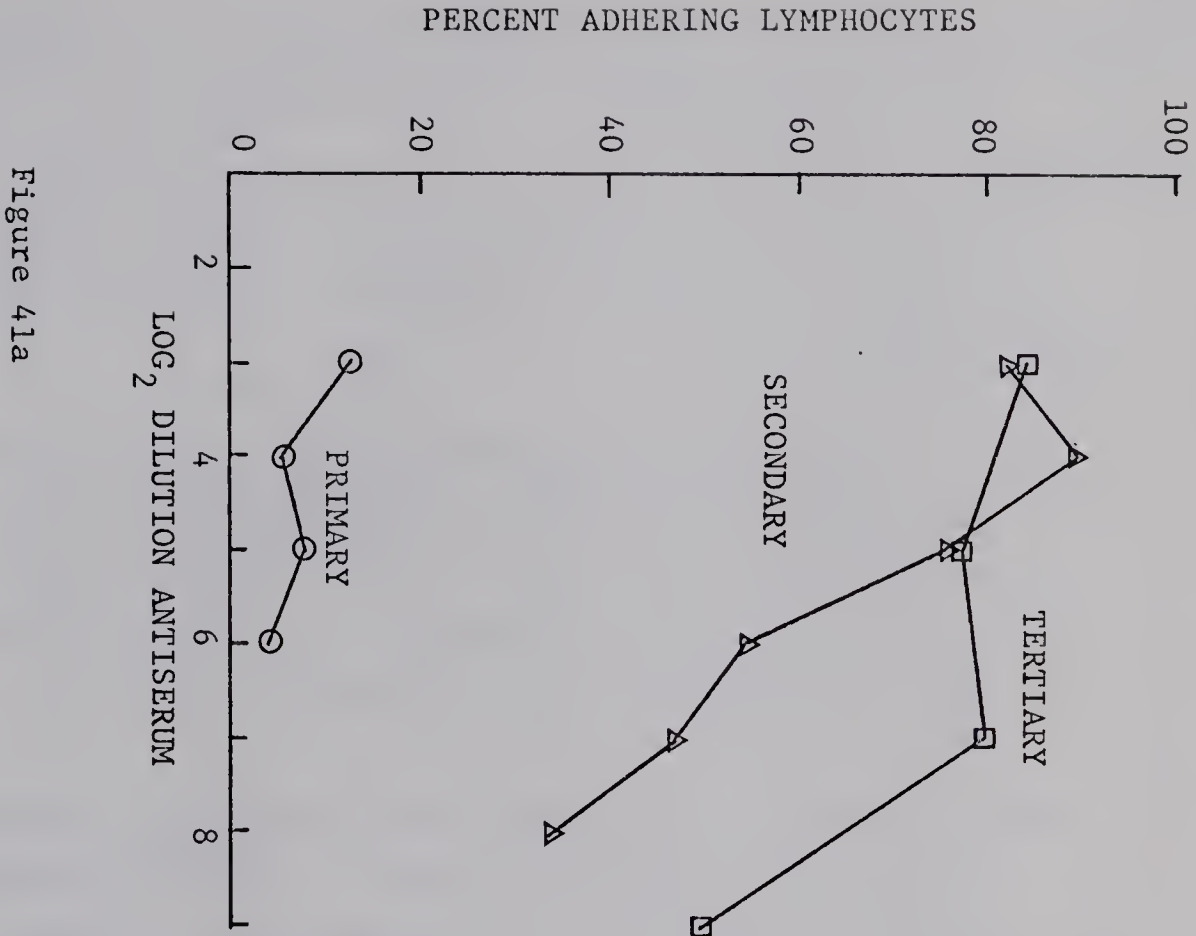








Figure 42. Allofixation (AF) and Hemagglutination (HA) titers of primary, secondary and tertiary antisera.

- (a) Allofixation and hemagglutination titers of unabsorbed anti- $B_2$  prepared in  $\underline{B^1}/\underline{B^{14}}$  and tested on  $\underline{B^2}/\underline{B^2}$  lymphocytes and erythrocytes.
- (b)
- (c) Allofixation and hemagglutination titers of unabsorbed anti- $B_{14}$  prepared in  $\underline{B^2}/\underline{B^2}$  and tested on  $\underline{B^{14}}/\underline{B^{14}}$  lymphocytes and erythrocytes. Three series of tests were performed with the primary and secondary antisera, but only one series of test was done with the tertiary antiserum.
- (d) Allofixation and hemagglutination titers of unabsorbed anti- $B_{14}$  prepared in  $\underline{B^1}/\underline{B^2}$  and tested on  $\underline{B^{14}}/\underline{B^{14}}$  lymphocytes and erythrocytes. Four series of tests were performed with the primary antiserum, two series for the secondary and only one series for the tertiary antiserum.

The allofixation test was performed at room temperature for 2 hours and at pH 8.5. The 50% cell adherence was taken as the end point for allofixation. The hemagglutination test was done at room temperature for 30 minutes.

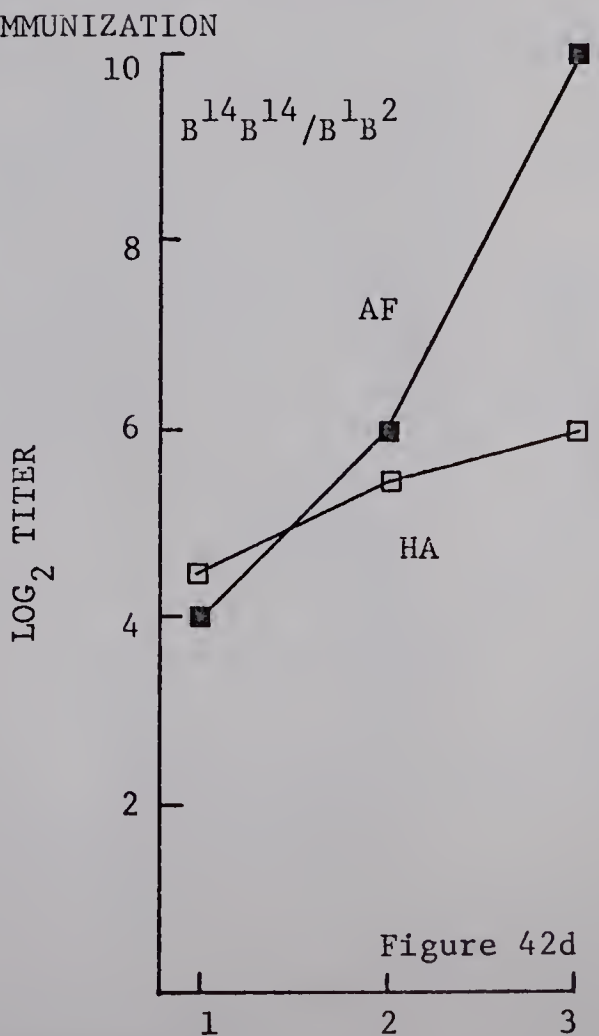
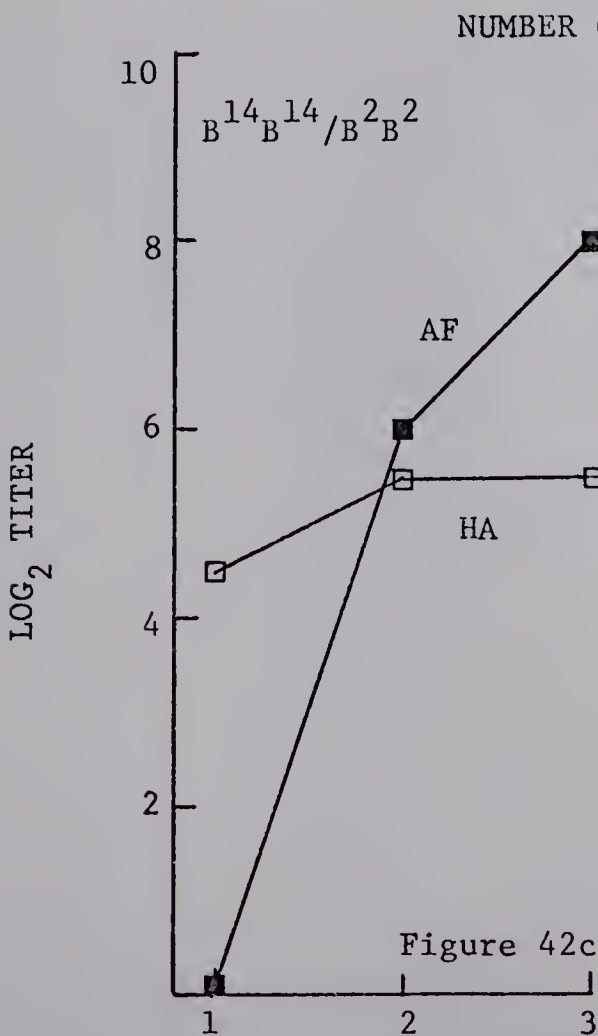
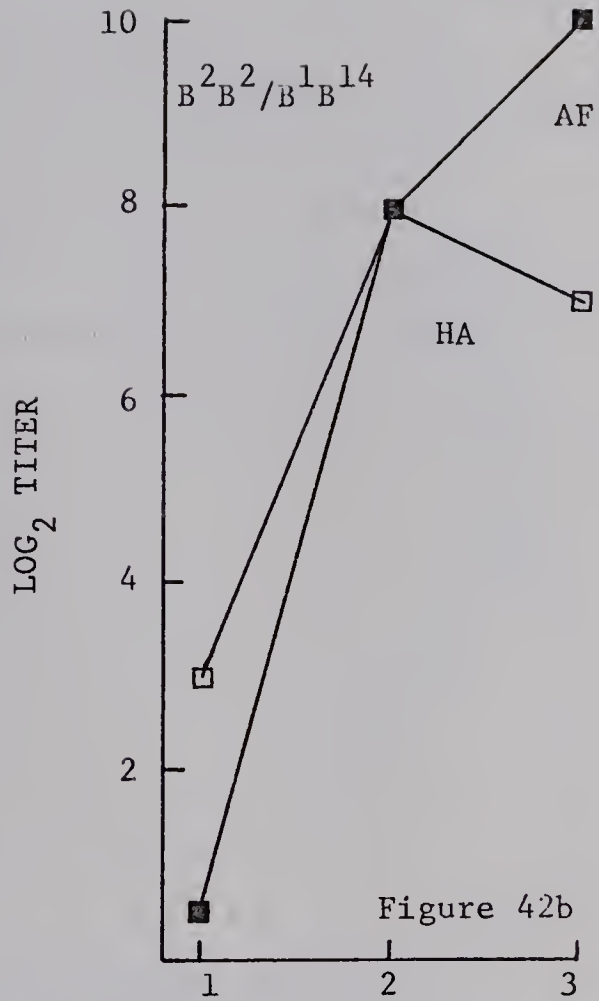
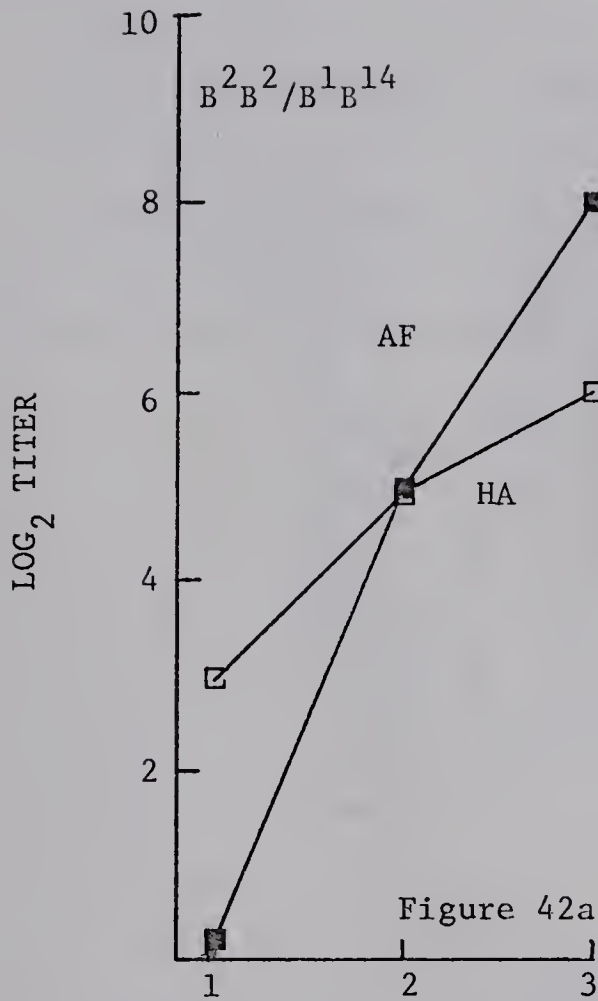






Figure 43. The effect on allofixation of treating the antiserum with rabbit anti-chicken IgG-serum or agamma chicken serum.  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  after treating at 37°C for 30 minutes with equal amounts of rabbit anti-chicken IgG-serum or agamma chicken serum. Two series of tests were done in each case at room temperature for 2 hours. The means of the two tests were taken.

Figure 44. The effect on allofixation of treating the antisera with mercaptoethanol (ME).  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested with unabsorbed anti- $B_{14}$  prepared in  $\underline{B}^2/\underline{B}^2$  and  $\underline{B}^1/\underline{B}^2$  birds after treating with equal amounts of 0.1 M mercaptoethanol at room temperature for 1 hour. Two series of the test were done at room temperature for 2 hours and at pH 8.5. Two primary and 2 hyperimmune antisera were employed in the tests.



PERCENT ADHERING LYMPHOCYTES

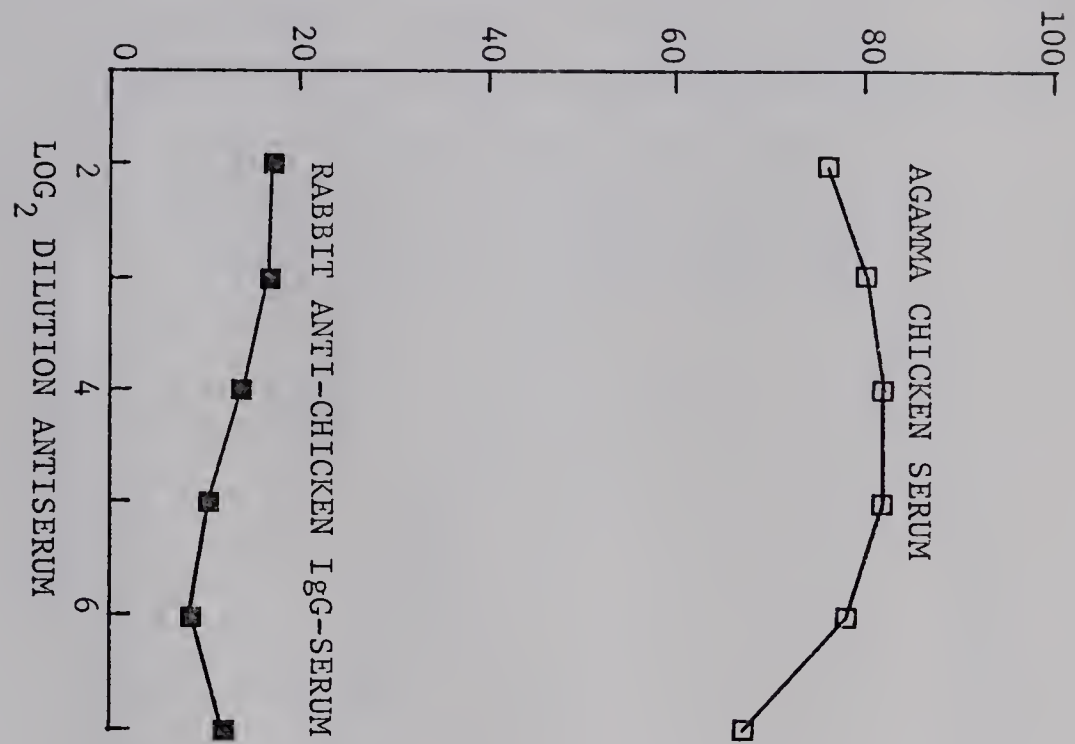


Figure 43

PERCENT ADHERING LYMPHOCYTES

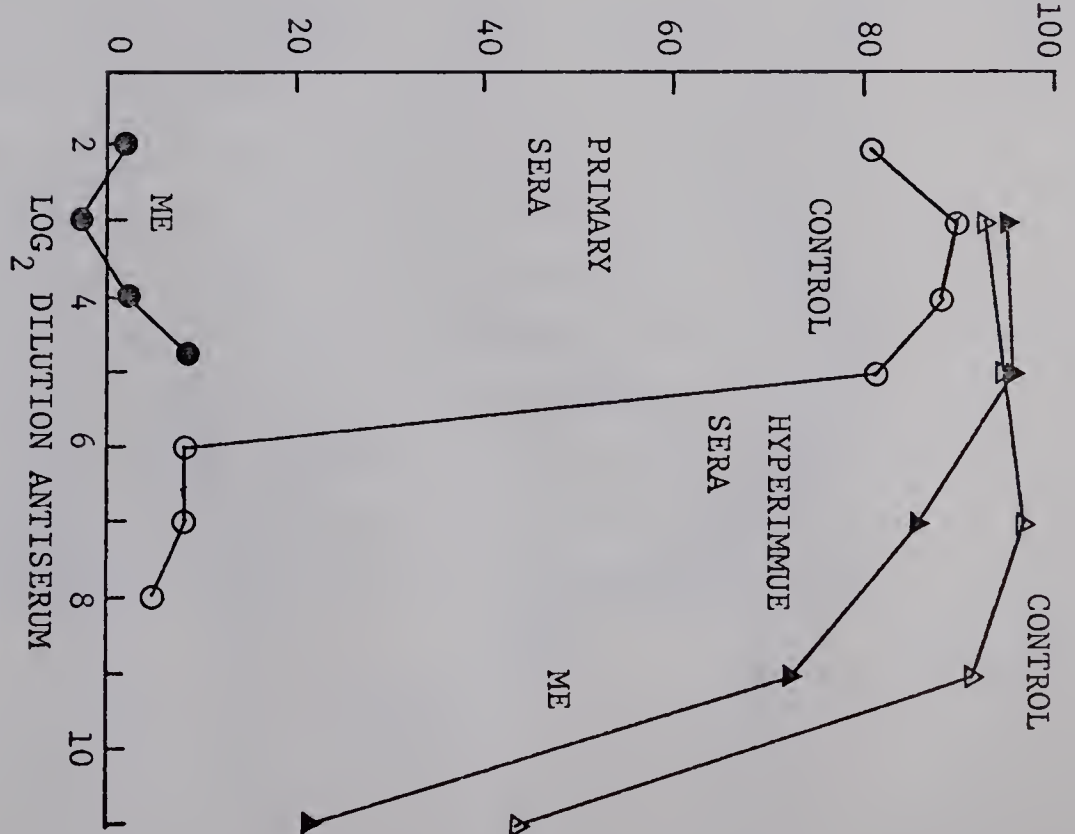


Figure 44





Figure 45. The effect of mercaptoethanol on the allofixation of primary and hyperimmune antisera. Duplicate tests were performed on  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with 2 primary and 4 hyperimmune antisera at room temperature for 2 hours and at pH 8.5. One, 3 and 5 mM of mercaptoethanol was added to the test system at the beginning of the experiments.

Figure 46. The adherence of chicken lymphocytes with untreated and heat-inactivated goat anti-chicken red cell-serum.  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were tested at different dilutions of the antiserum with or without the supplement of fresh chicken plasma at room temperature for 2 hours and at pH 8.5. Two series of tests were performed in each case and the lysis or cytotoxicity of cells was detected under phase contrast microscope. Complete lysis of cells was detected at antiserum dilutions smaller than 1/8; cytotoxicity was detected from 1/8 to 1/512 dilutions of the untreated antiserum, but was detected from 1/8 to 1/125 dilutions of the heat-inactivated antiserum.

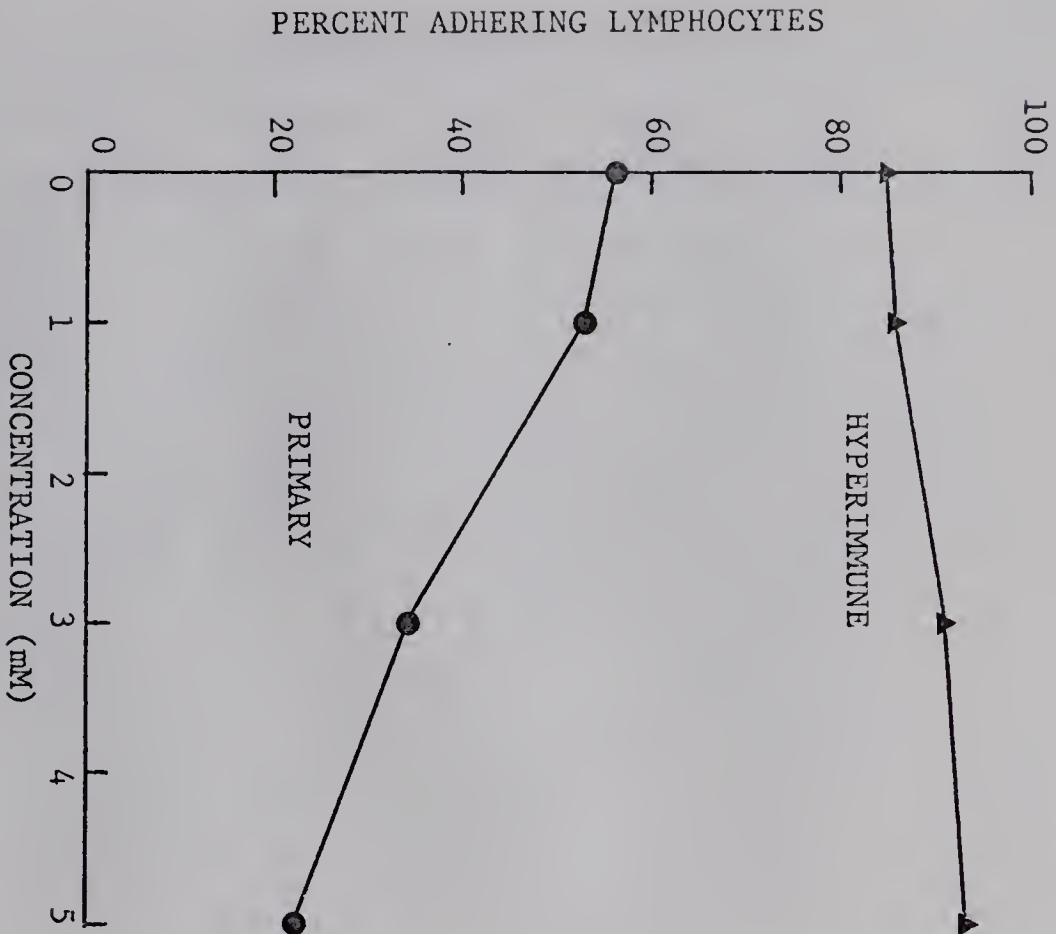


Figure 45

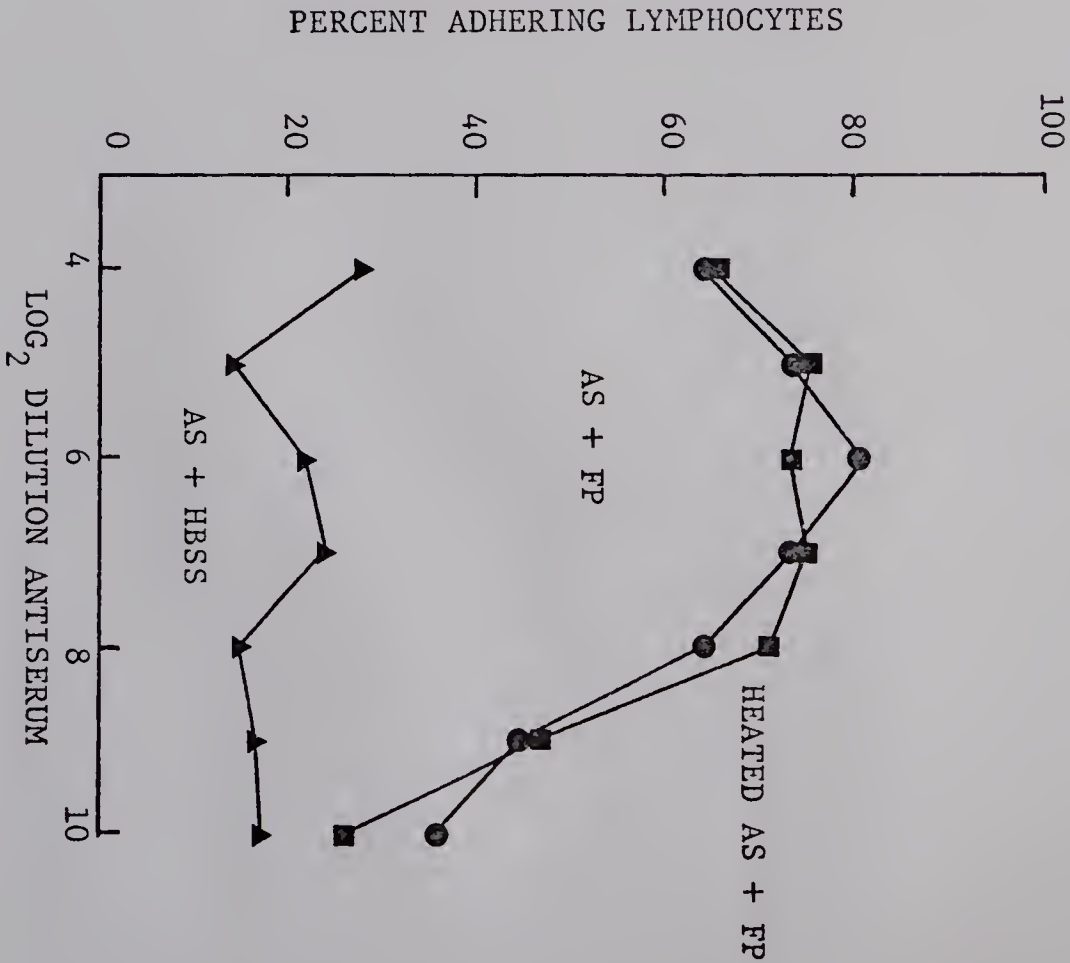


Figure 46







Figure 47. The effect of fresh mouse plasma (MFP) on the adherence of mouse lymph node cells induced by chicken anti-mouse red cell-serum and fresh chicken plasma. Lymph node cells of Balb/C or CBA mice were used in the tests. The tests were done at room temperature for 2 hours and at pH 8.5 with or without the presence of fresh mouse plasma. Two series of tests were performed in the presence of 25% MFP and three series of tests were done in the presence of 1% MFP. Twenty five percent of fresh chicken plasma was added into the test system in all cases.

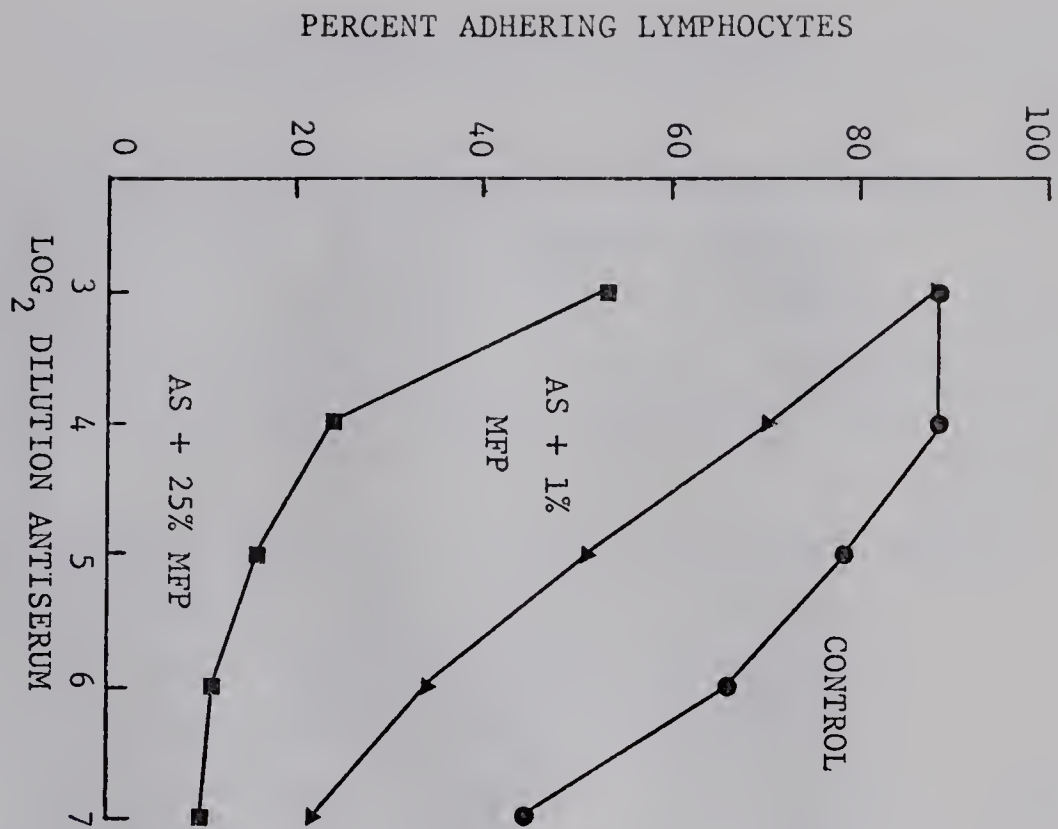


Figure 47





Figure 48. Cell adherence induced with plant lectins.

- (a) Cell adherence induced with Concanavalin A (Con A) in the presence of serum or plasma.  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes were tested with Con A at room temperature for 2 hours and at pH 7.0. Twenty five percent fresh chicken plasma or agamma chicken serum was used to prevent the aspecific adherence in HBSS.
- (b) Cell adherence induced with Phytohemagglutinin (PHA) in the presence of serum or plasma.  $\underline{B}^2/\underline{B}^{14}$  lymphocytes were tested with PHA-P at room temperature for 2 hours and at pH 7.0. One percent of fresh chicken plasma or agamma chicken serum was used to inhibit the aspecific adherence in HBSS. Agglutination may be associated with cell adherence in some of these tests.
- (c) Cell adherence test with Pokeweed mitogen (PWM).  $\underline{B}^2/\underline{B}^{14}$  lymphocytes were tested with PWM at room temperature for 2 hours and at pH 7.0. One percent fresh chicken plasma was used to inhibit the aspecific adherence in HBSS.



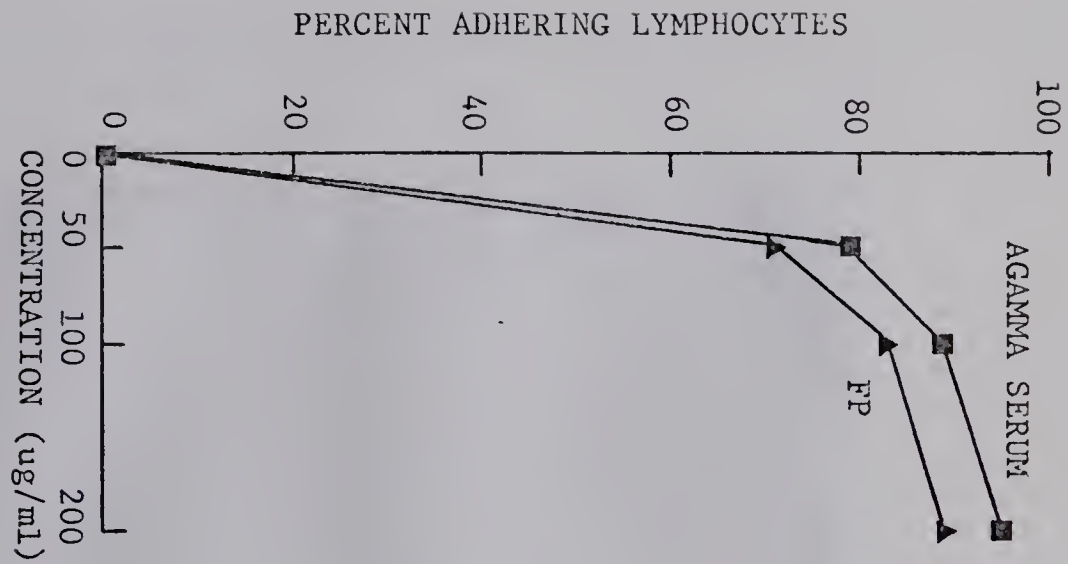


Figure 48a

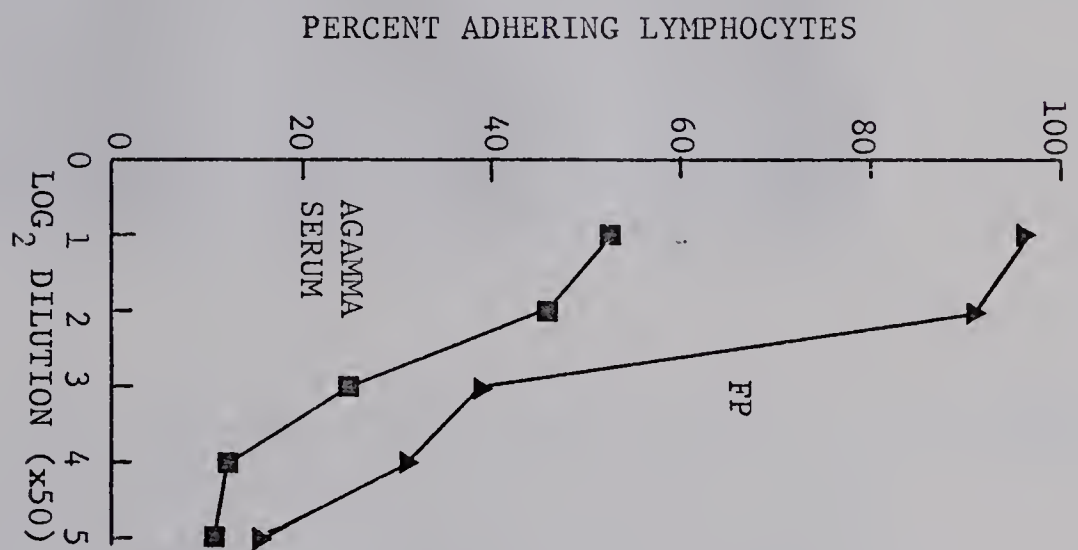


Figure 48b

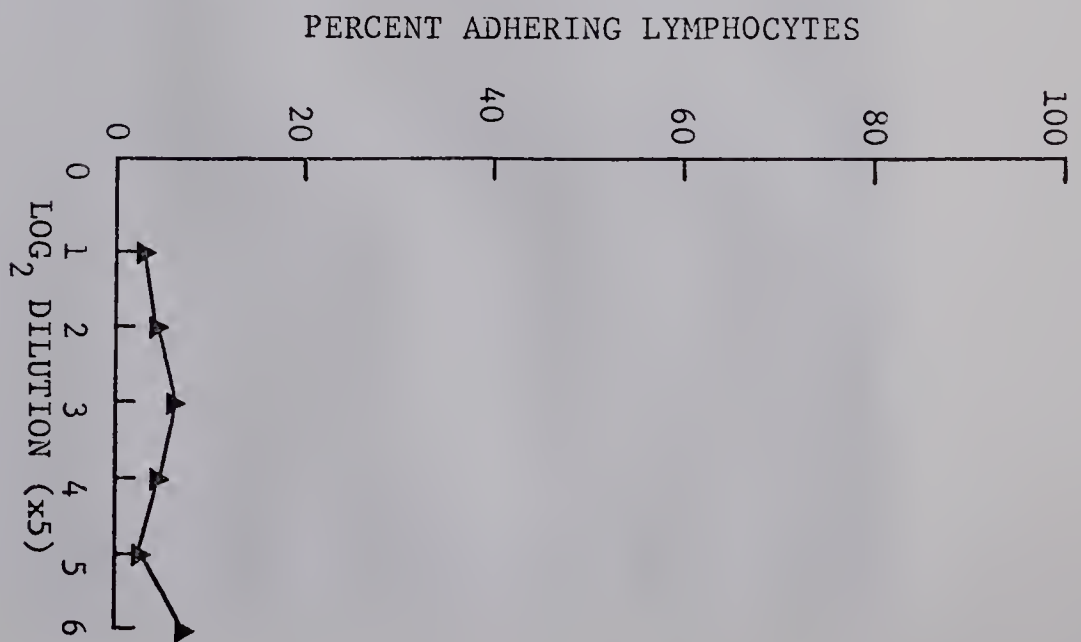


Figure 48c





Figure 49a. The adhering lymphocytes on the surface of a plastic petri dish.  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were fixed by 100 ug/ml Con A at room temperature for 2 hours at pH 7.0 and in the presence of 25% agamma chicken serum.

(Magnification 5000x)

Figure 49b. The adhering lymphocytes on the surface of a plastic petri dish.  $\underline{B}^2/\underline{B}^{14}$  lymphocytes were fixed by 1/100 dilution of PHA-P at room temperature for 2 hours at pH 7.0 and in the presence of 1% agamma chicken serum.

(Magnification 5000x)

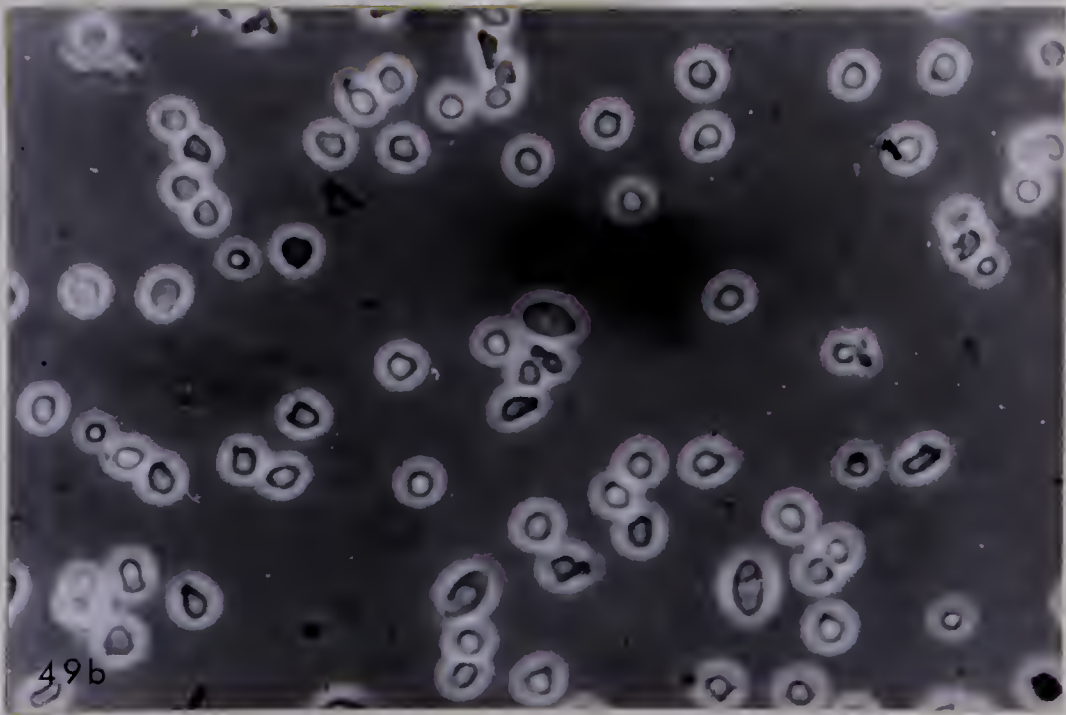








Figure 50. The effect of pH on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^2/\underline{B}^{14}$  lymphocytes with 200 ug/ml Con A at room temperature for 1 and 2 hours in the presence of 25% fresh chicken plasma or agamma chicken serum. The pH was adjusted with 0.1 N NaOH or HCl.

Figure 51. The effect of concentration of Con A on Con A-induced cell adherence.  $\underline{B}^2/\underline{B}^{14}$  lymphocytes were tested at room temperature for 1 and 2 hours in the presence of 25% fresh chicken plasma or agamma chicken serum at pH 7.0.

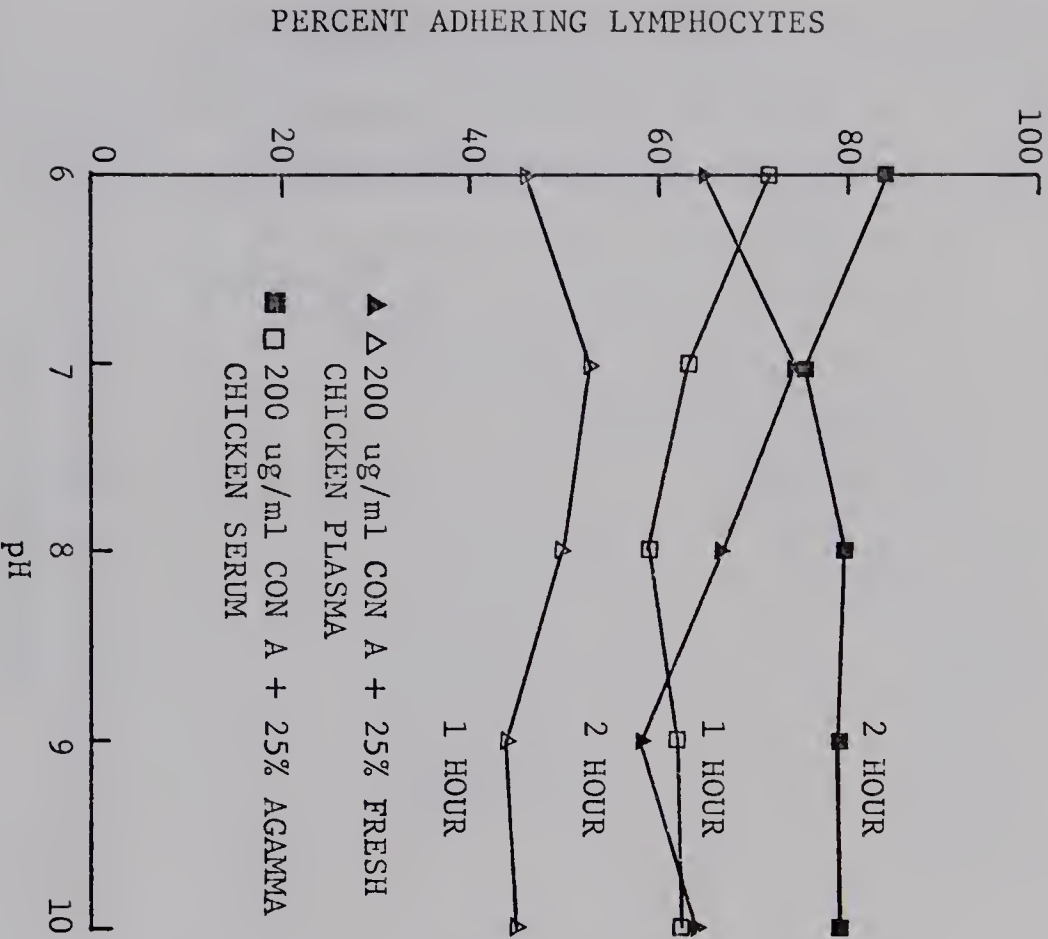


Figure 50

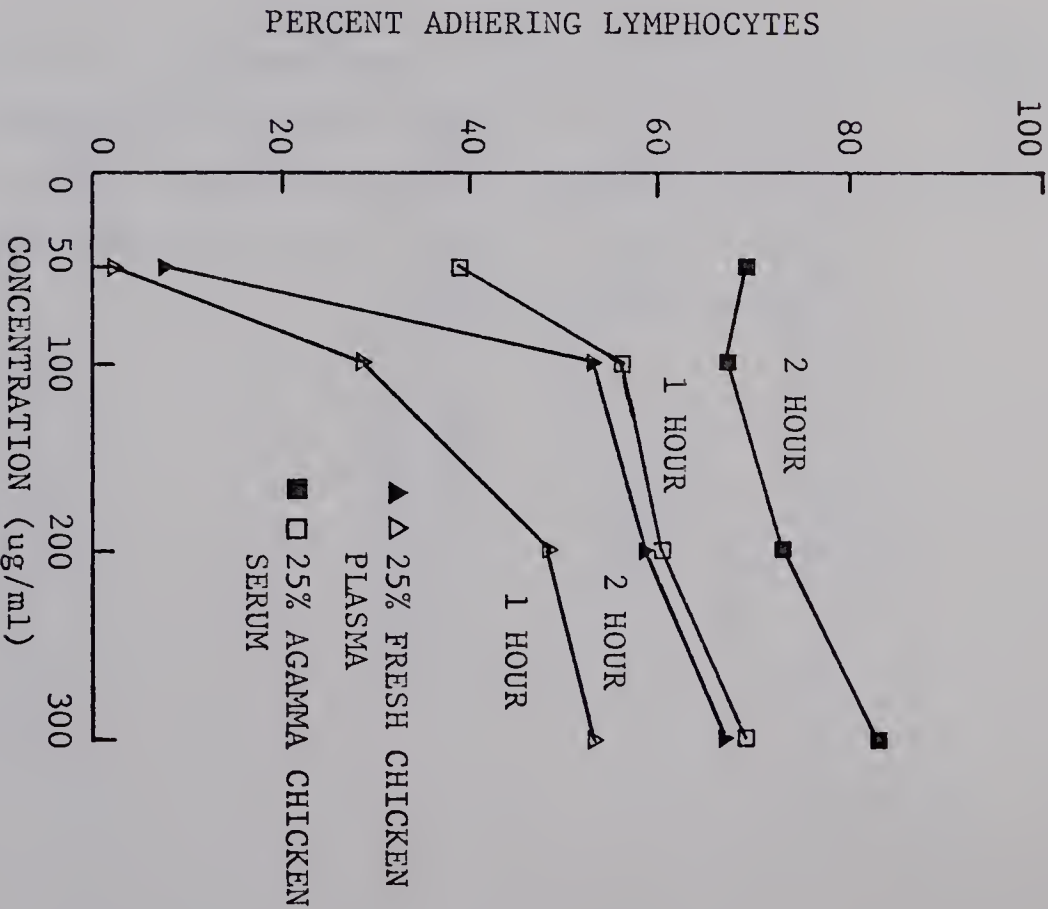


Figure 51





Figure 52. The effect of serum concentration on Con A-induced cell adherence.  $\underline{B}^2/\underline{B}^{14}$  lymphocytes were tested with 25, 50 and 100 ug/ml of Con A at room temperature for 1 hour at pH 7.0.

Figure 53. The effect of cell concentration on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^2/\underline{B}^2$  lymphocytes with 100 and 200 ug/ml Con A at room temperature for 1 and 2 hours at pH 7.0 in the presence of 25% agamma chicken serum.



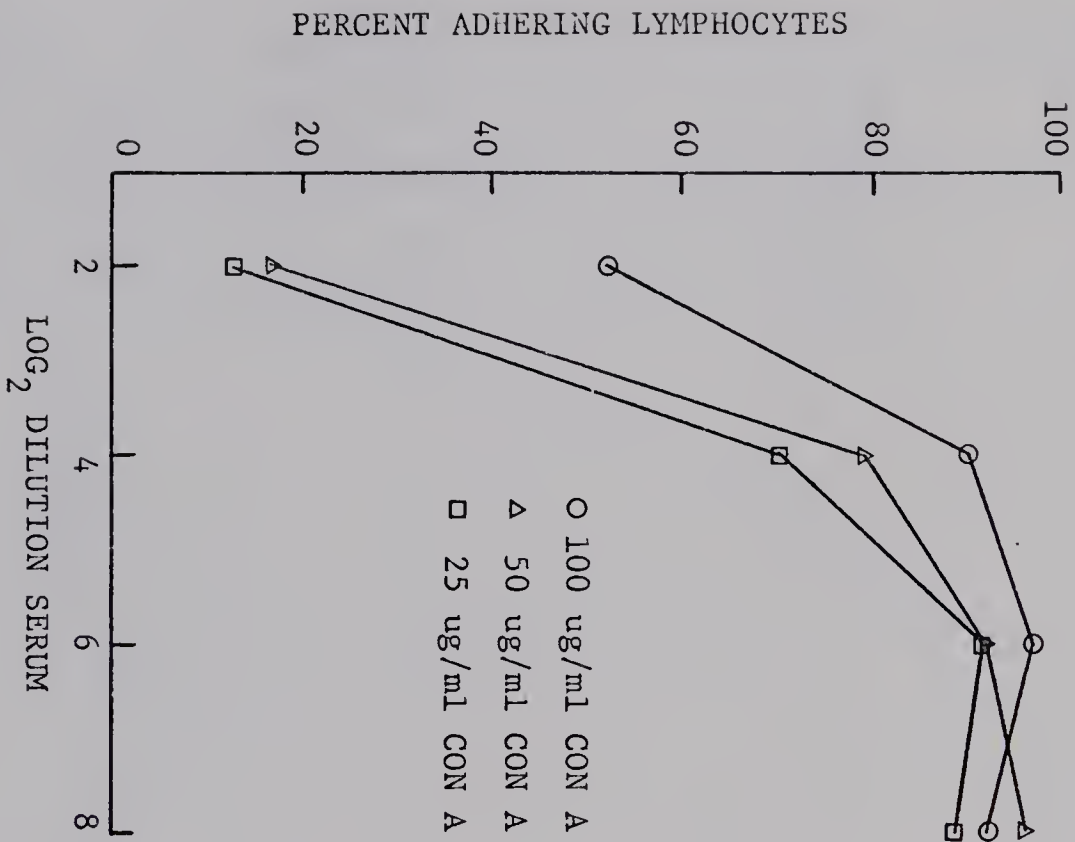


Figure 52

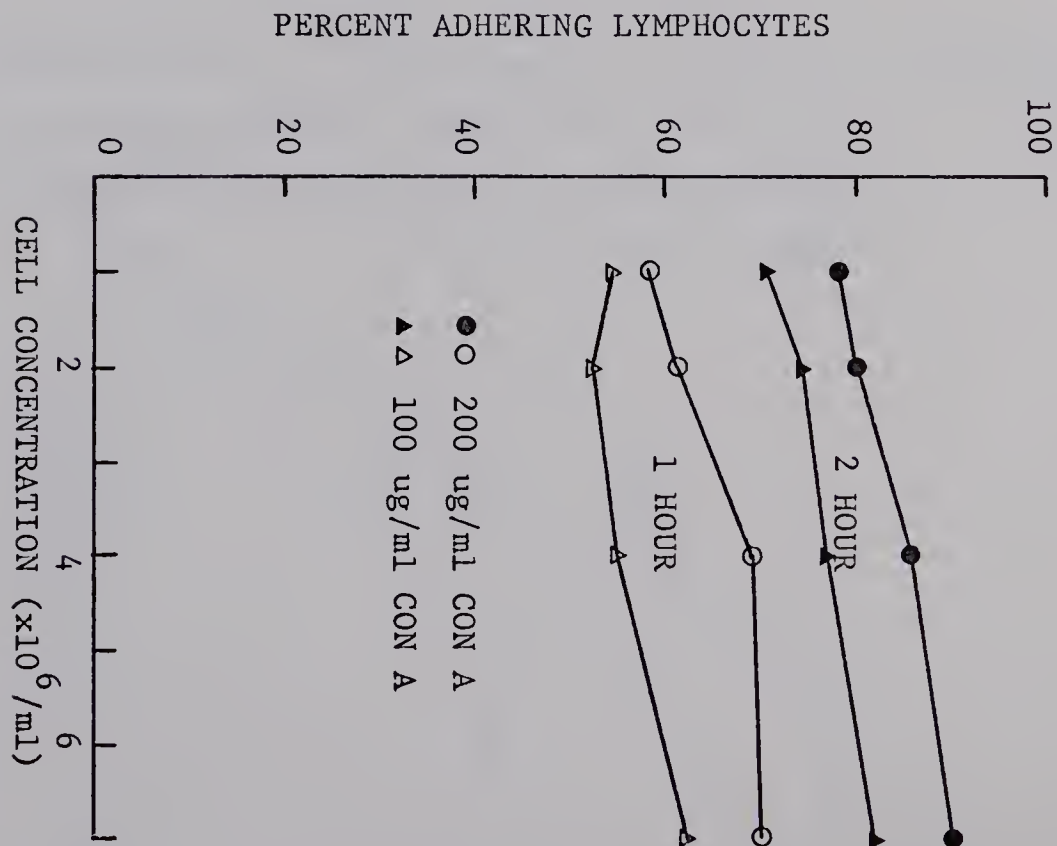


Figure 53





Figure 54. The effect of time of incubation on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^{15}/\underline{B}^{15}$  lymphocytes with 50 ug/ml Con A for a period of 90 minutes in the presence of 1% fresh chicken plasma. Counts were taken at 15 minute intervals for the first hour of tests.

Figure 55. The effect of temperature on Con A-induced cell adherence. Two series of duplicate test were performed on  $\underline{B}^2/\underline{B}^{14}$  lymphocytes for 1 and 2 hours in the presence of 25% agamma chicken serum at pH 7.0.

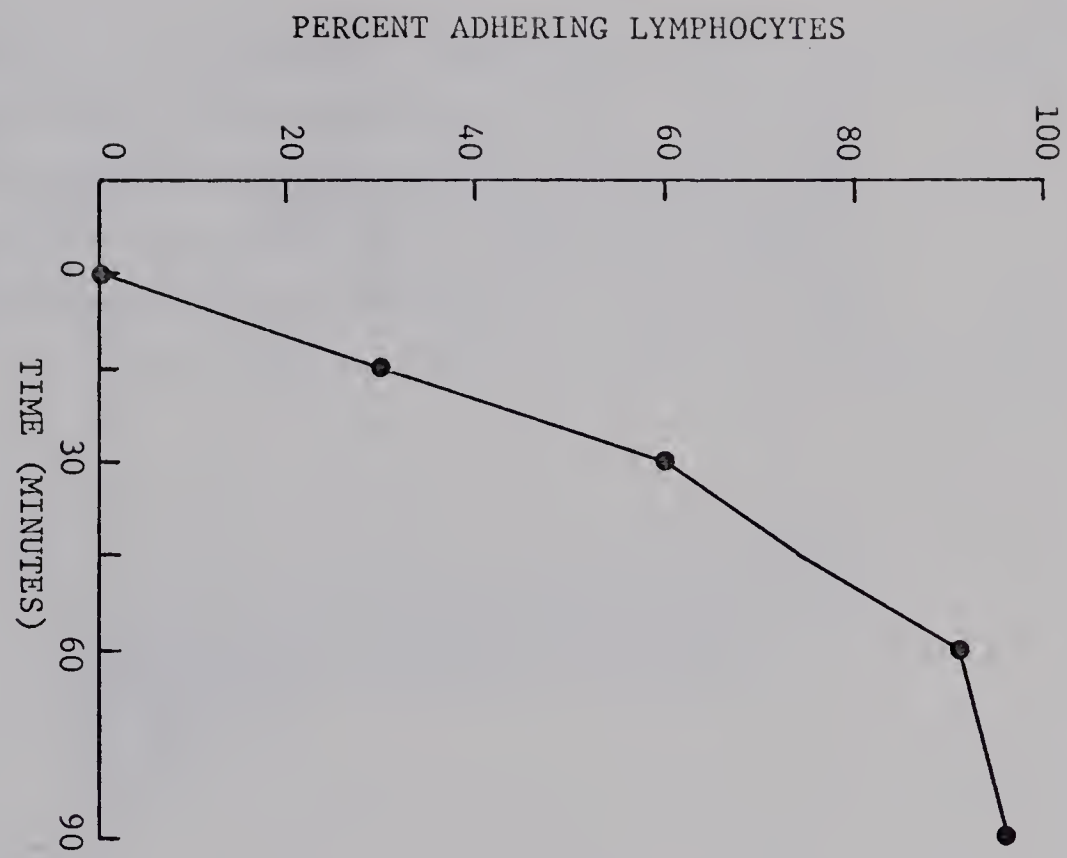


Figure 54

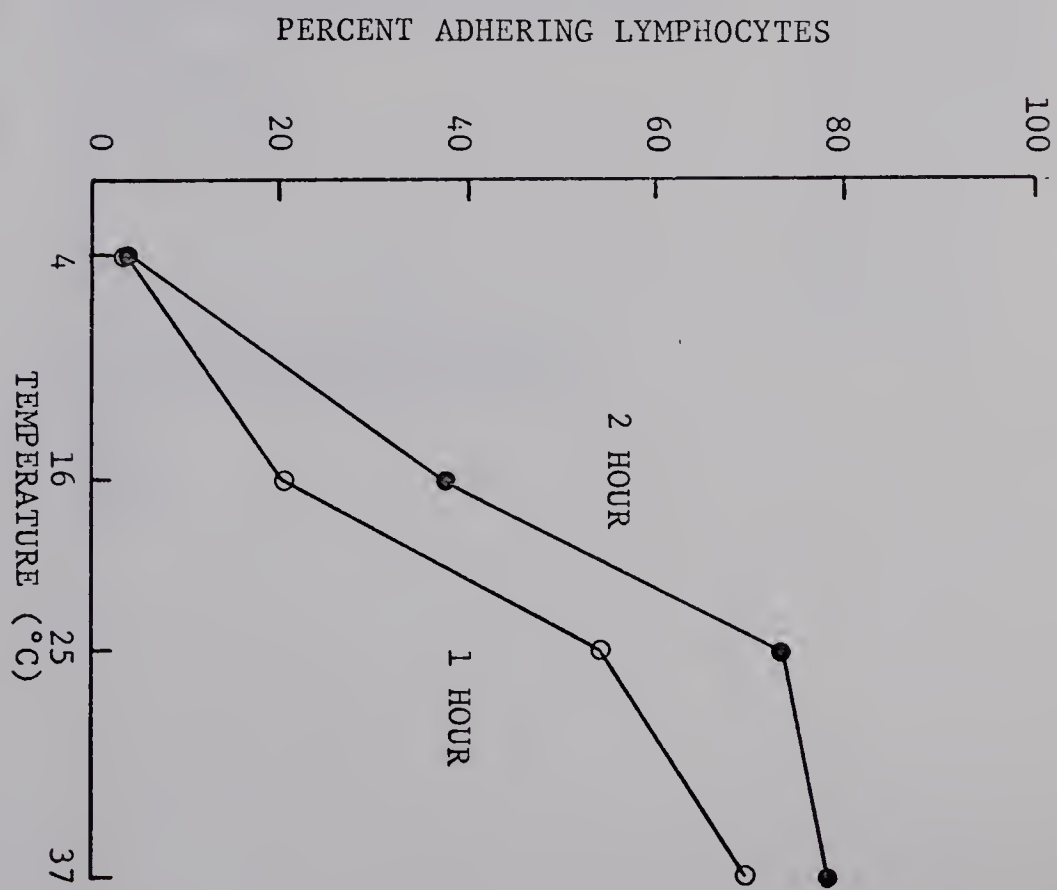


Figure 55







Figure 56. The effect of specific inhibitors on Con A-induced cell adherence.  $\underline{B}^2/\underline{B}^2$  lymphocytes were tested with 50, 100 and 200 ug/ml Con A with or without the addition of 0.05 M specific inhibitors. The tests were run at room temperature for 2 hours in the presence of 25% agamma chicken serum.

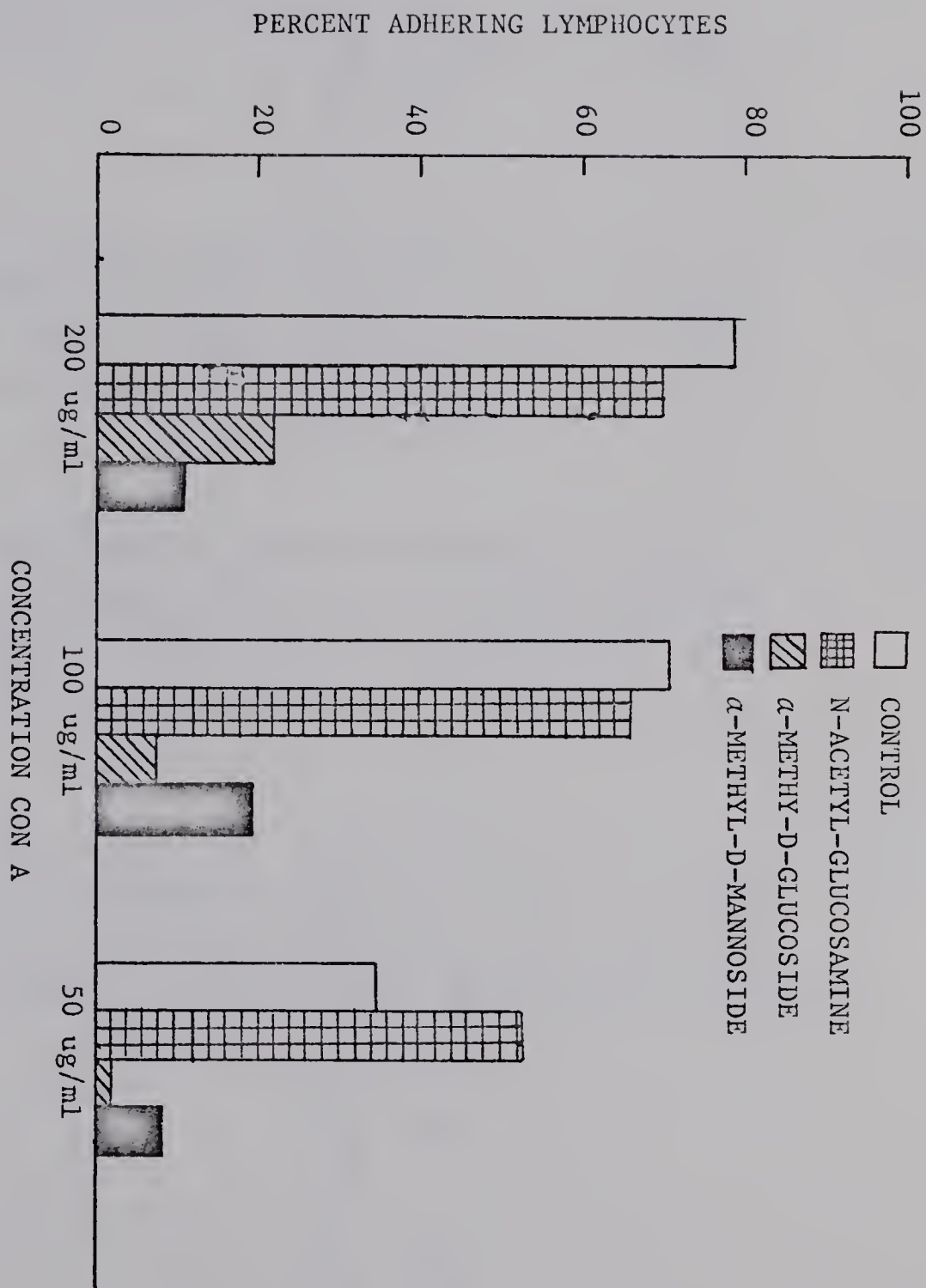


Figure 56





Figure 57. The effect of metabolic inhibitors, sodium cyanide and iodoacetamide, and chelating agent, EDTA, on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^1/\underline{B}^1$  lymphocytes with 100 ug/ml Con A at room temperature for 2 hours in the presence of 25% agamma chicken serum at pH 7.0.

Figure 58. The effect of metabolic inhibitor, 2-deoxy-D-glucose, on Con A-induced cell adherence. Duplicate tests were done on  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with 100 ug/ml Con A at room temperature for 2 hours in the presence of 25% agamma chicken serum at pH 7.0.

Figure 59. The effect of chloramphenicol on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with 100 ug/ml Con A at room temperature for 2 hours in the presence of 25% agamma chicken serum at pH 7.0.

Figure 60. The effect of cycloheximide on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with 100 ug/ml Con A at room temperature for 2 hours in the presence of 25% agamma chicken serum at pH 7.0.



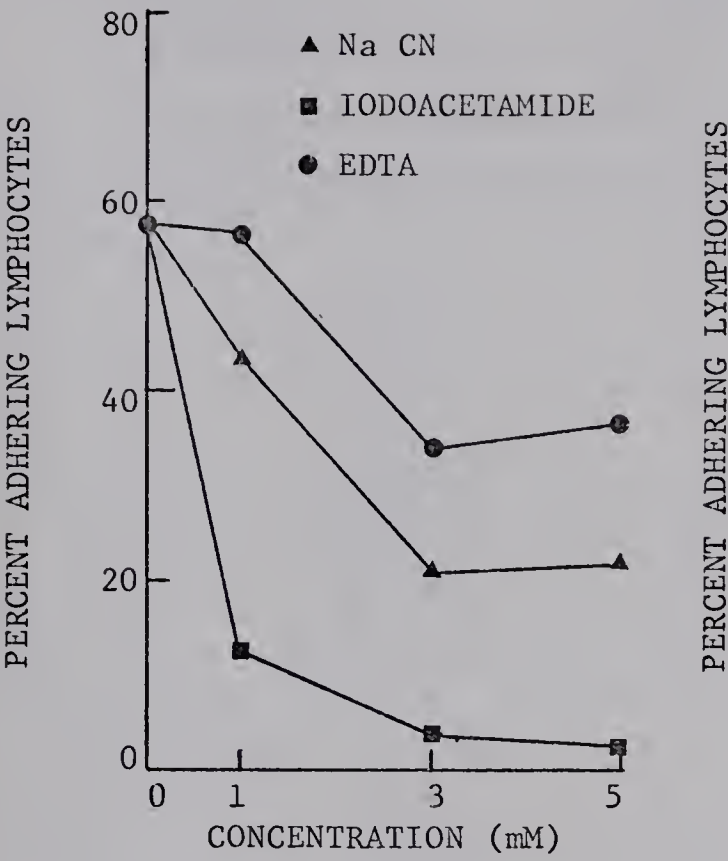


Figure 57

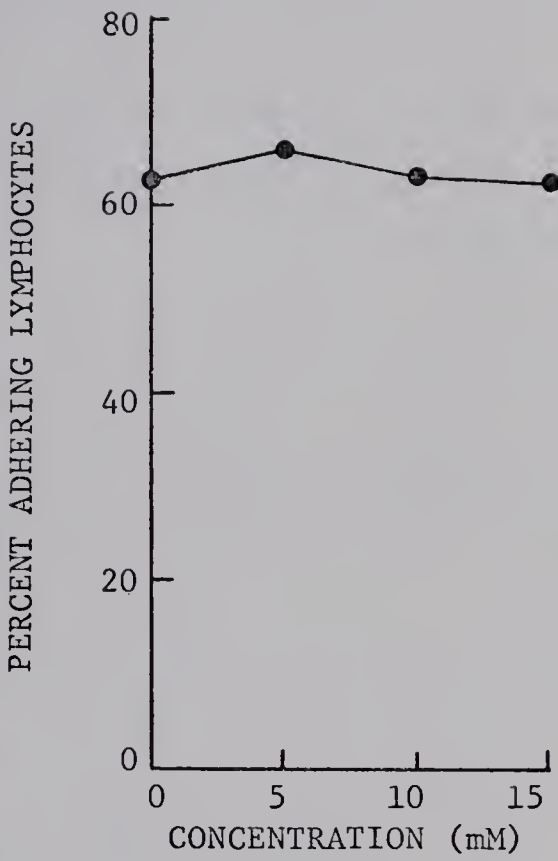


Figure 58

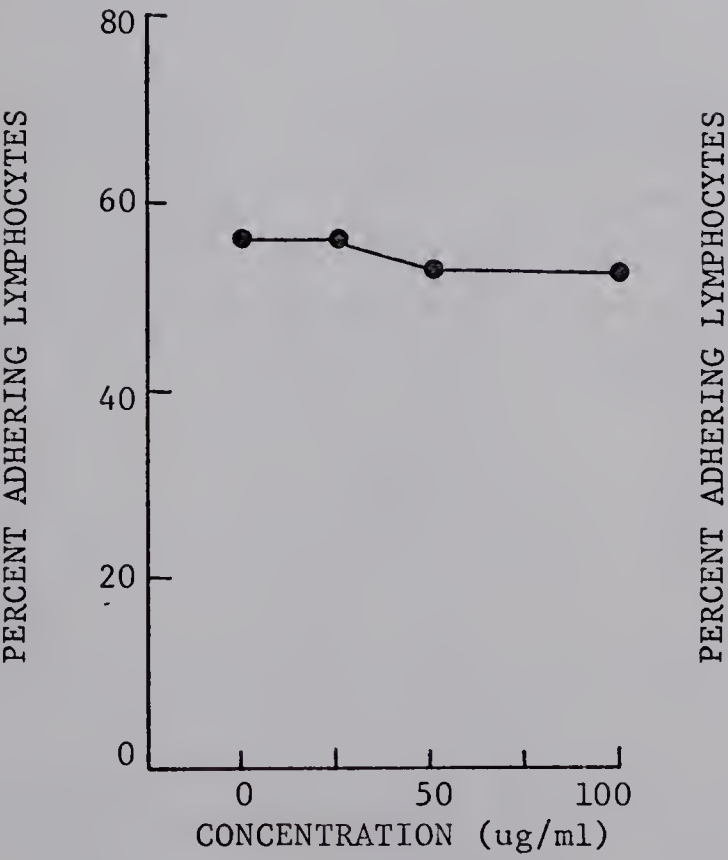


Figure 59

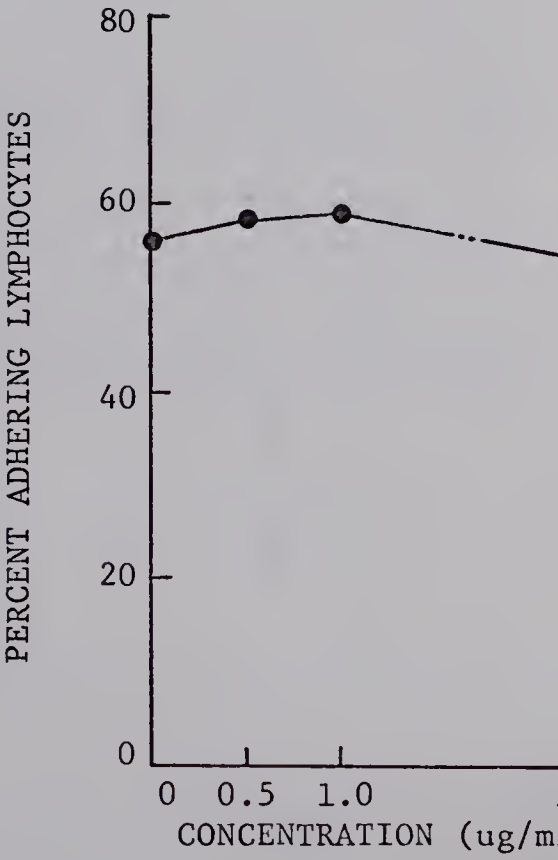


Figure 60





Figure 61. The effect of hydrocortisone on Con A-induced cell adherence. Duplicate tests were performed on  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes with 100 ug/ml Con A at room temperature for 2 hours in the presence of 25% agamma chicken serum at pH 7.0.

Figure 62. The effect on allofixation of treating the lymphocytes with Con A.  $\underline{B}^{14}/\underline{B}^{14}$  lymphocytes were treated with 50, 100 and 200 ug/ml Con A at room temperature for 2 hours. Duplicate tests were performed on these treated lymphocytes with a specific anti- $B_{14}$  serum at room temperature for 2 hours at pH 8.5. The controls were run under the same conditions with the omission of antiserum.

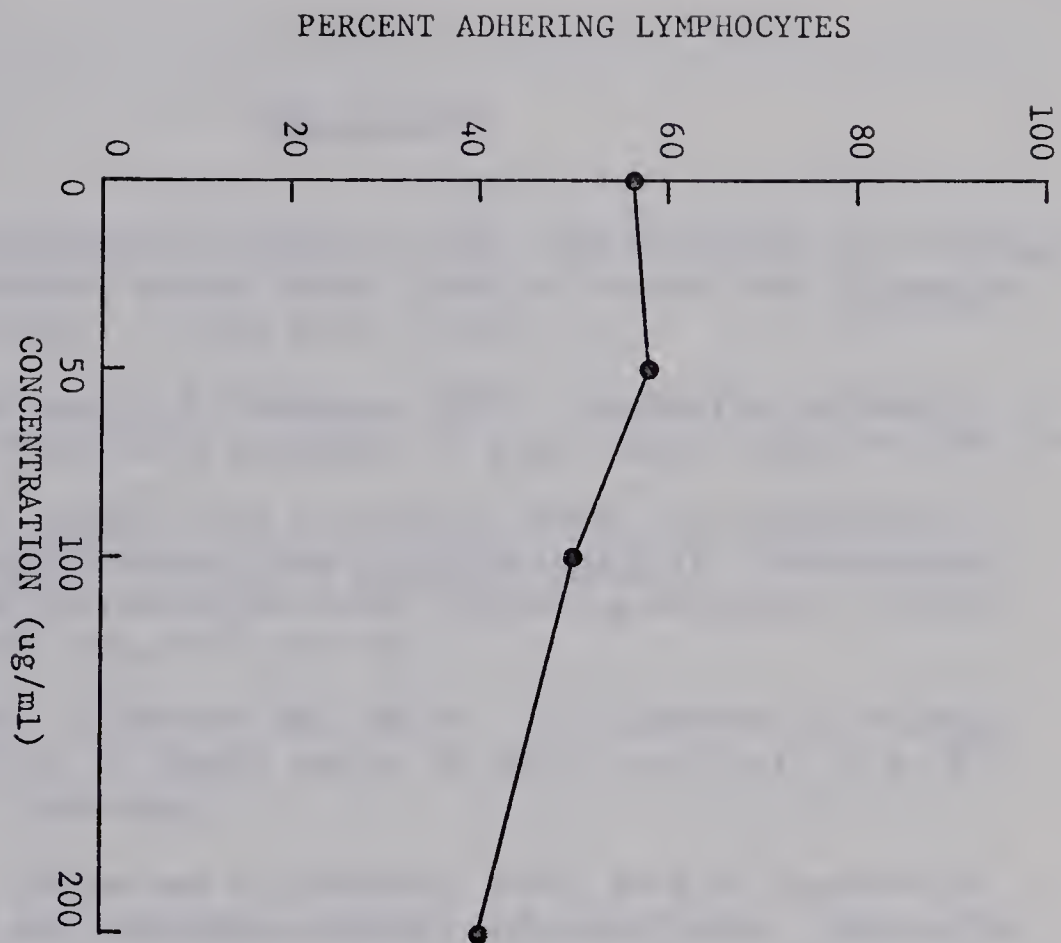


Figure 61

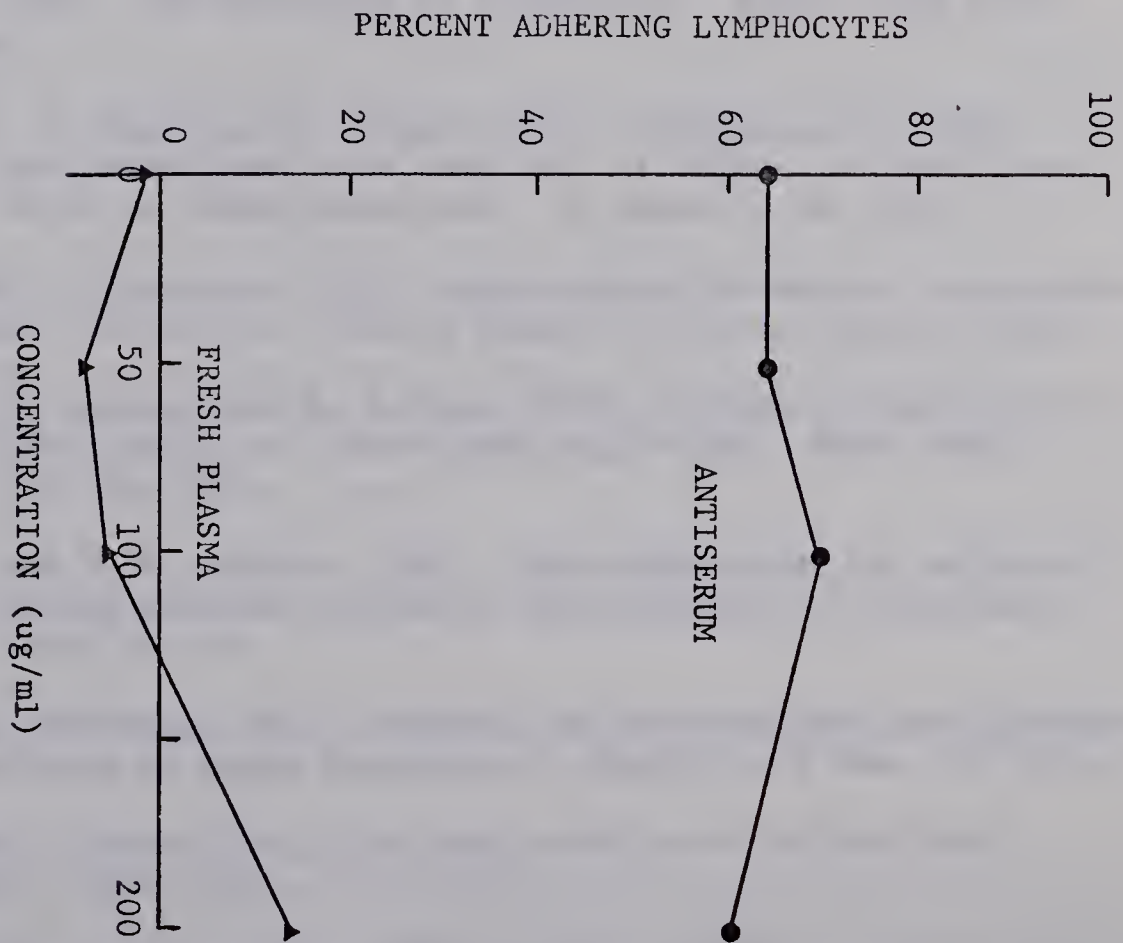


Figure 62





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